pH-Dependent Proton Absorption in Chymotrypsin. Small Molecule Interactions

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Abstract: Inhibitor binding to α-chymotrypsin results in proton uptake, which is not observed with the zymogen or phosphorylated enzyme. A single ionizing group of apparent $pK_a = 8.8$ is involved in the binding and proton uptake processes. At a given pH proton absorption exhibits a saturation phenomenon which exactly parallels substrate binding, providing direct confirmation of the intimate relationship between the enzyme's state of protonation and its ability to bind small molecules at high pH. pH recorder and spectrophotometric pH indicator dye techniques were used to observe changes in the enzyme's state of protonation. Models to account for all observed high pH chymotrypsin phenomena are proposed, along with detailed possible mechanisms for the active site conformational change.

One of the more interesting problems encountered recently in enzyme mechanism chemistry has been to explain in detail the inhibition of specific substrate binding by chymotrypsin at alkaline pH.[2-4] A "conformational change" has been proposed[3,5,6] and, in more precise terms, an intramolecular competitive inhibition at the active site.[4] That the binding of a neutral molecule to the enzyme is pH dependent necessarily implies that a reversible change in the enzyme's state of protonation occurs upon binding. The particular ionizing group implicated in the chymotrypsin mechanism is the α-amino group of isoleucine-16 at the N terminus of the B chain, which is liberated upon activation of the zymogen to δ-chymotrypsin.

The approach and reaction of reagents which derivatize noncatalytic groups in the active site are also inhibited at high pH, *e.g.*, the alkylation of the thioether group of methionine-192 by benzyl bromide.[7] Other techniques reveal changes in properties of the enzyme near pH 9: comparing active enzyme to zymogen, the optical rotatory and uv difference spectra are altered.[6,8] Similar changes could be induced upon phosphorylation or acylation of the active enzyme, but not in any way with the zymogen. Rapid spectral observations seem to indicate that binding involves two steps at high pH but only one step at low pH.[9] Phosphorylated chymotrypsin exhibits one less titratable group (up to about pH 10) than does the active enzyme, indicating that phosphorylation shifts the pK_a of the ionizing group from below 9 to above 10.5.[5]

If binding occurs preferentially to a protonated form of chymotrypsin, saturation with substrate should result in proton uptake equal to the amount of enzyme initially deprotonated at a given pH. Indeed, for several years rapid initial proton absorption has been reported to accompany acylation reactions of chymotrypsin,[10-13] but no attempt was made to separate the effects due to binding (K_s) from those due to acylation (k_2). Recent studies indicate that binding alone is sufficient to cause proton absorption by chymotrypsin at high pH,[4,5] and that no further changes in the enzyme's state of protonation should result from the acylation (k_2) step. These predictions have been followed up in detail experimentally, using several techniques for observing small pH changes, four different enzyme forms, and a variety of substrates and inhibitors. Preliminary results of this work have been communicated.[14] The completed research gives rise to a more meaningful interpretation of the enzyme's behavior in relation to its structure at high pH.

Experimental Section

Materials. All enzyme solutions were 0.1 m*M* unless otherwise noted.

α-Chymotrypsin was a Worthington product (three times crystallized, salt-free), Lot CDI-6HA. Stock solutions were prepared in 0.1 *M* KCl–water solution, and the active enzyme concentration was determined by spectrophotometric titration with N-*trans*-cinnamoylimidazole[15] immediately prior to use.

Chymotrypsinogen A was a Worthington product (five-times recrystallized, lyophilized, chromatographically homogeneous, salt-free). Lot CGC-6EA was used in the equilibrium dialysis experiments with benzyl alcohol, and Lot CG-4GA was used in the "blank" reactions with the proton absorption experiments. Stock solutions were prepared just before use, and the concentration of protein determined spectrophotometrically at 280 nm, using an extinction coefficient of $4.7 \times 10^4\ M^{-1}\ cm^{-1}$ [16] and mol wt 25,000.

δ-Chymotrypsin was a Worthington product (three-times crystallized, lyophilized, salt-free), Lot CDD-6031. Stock solutions were prepared and the active enzyme concentration was determined exactly as with α-chymotrypsin above.

Diisopropylphosphofluoridate-treated α-chymotrypsin was a Worthington product (three-times crystallized, salt-free), Lot CDDFP-6JA. Stock solutions were prepared exactly as with α-chymotrypsin, and were shown by specific substrate assay[17] to

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contain less than 1% active enzyme. This species is abbreviated DIP-α-chymotrypsin hereafter.

p-Nitrophenyl N-carbobenzyloxy-L-tyrosinate was a Mann Biochemicals product, used as delivered, mp 156° (lit.[17] mp 157°). Stock solutions of 1.0 and 0.1 m*M* were prepared for use in rate assays.

N-*trans*-Cinnamoylimidazole was synthesized as previously described[15] and was recrystallized from dry *n*-hexane, mp 132° (lit.[15] mp 133°).

N-*trans*-Indoleacryloylimidazole was synthesized as previously described[18] and was recrystallized from dry acetonitrile, mp 197–199° (lit.[18] mp 199–200°).

N-Acetyl-L-tryptophanamide was a Cyclo Chemical Co. product, Lot R-4739, Grade I, used as delivered: mp 190–192° (lit.[19] mp 192–193°).

N-Acetyl-D-tryptophanamide was a Cyclo Chemical Co. product, Lot K-5490, Grade I, used as delivered: mp 195–196° (lit.[19] mp 192–193°).

Benzyl alcohol was a Fischer Certified reagent, was purified according to Weissberger,[20] and was kept sealed under nitrogen.

Phenolphthalein was a Conray Products Co. reagent, Lot 10442A, of ACS purity. The stock solution contained 53 mg in 100 ml of ethanol–water (1:1, v/v).

Thymolsulfonphthalein was a Baker Analyzed reagent, Lot 3-332, of ACS purity. The stock solution contained 100 mg in 250 ml of water.

Acetonitrile, a Mallinckrodt "Nanograde" material, was used as an inhibitor and in the preparation of most substrate solutions without further purification or drying.

Techniques and Kinetic Runs. All melting points were observed with a capillary tube and stirred oil bath apparatus and are reported without correction. A Cary Model 14 recording spectrophotometer equipped with a thermostated cell compartment was used for spectra determinations and kinetic runs involving the pH-indicator dyes.

All enzyme solutions contained 0.1 *M* KCl as supporting electrolyte, were unbuffered, and were kept at pH 4–5 at 5°. The size and specificity of inhibitors employed in this study differ widely, as indicated by their K_i values at pH 7.9: acetonitrile (830 m*M*), benzyl alcohol (10 m*M*), and N-acetyltryptophanamide (D, 2.3 m*M*; L, 6 m*M*). Acetonitrile and benzyl alcohol were added to enzyme solutions without dilution; all other substrates or inhibitors were prepared in concentrated stock solutions of acetonitrile or methanol–water just prior to use. Addition of inhibitor to enzyme solution did not alter the total volume more than 1–5%, except in the case of acetonitrile which required volume changes up to 10% under saturating conditions at high pH, *i.e.*, 1 ml of CH_3CN/10 ml of enzyme solution.

The binding of benzyl alcohol to chymotrypsinogen A was studied *via* equilibrium dialysis.[21,22] Dialysis tubing (No. 20, Visking Corp., Chicago) was prepared as previously described,[21] then boiled 2 hr to remove additional uv-absorbing components. Five milliliters of enzyme solution was dialyzed against 10 ml of buffer. Controls omitting enzyme ensured that equilibrium had been attained within 36 hr at 25°, whether inhibitor was initially added inside or outside the dialysis bag. Spectral determination of benzyl alcohol in the presence of enzyme required difference spectrophotometry techniques. These data were proven accurate by preparing benzyl alcohol solutions of 10.0 and 10.2 m*M* (determined from individual spectra), then adding enzyme to each and determining the difference spectrum. The latter result agreed with the former within 10% or less, *i.e.*, a difference of 0.20 ± 0.02 m*M*.

A sensitive recording pH meter was used for most of the proton absorption studies.[13,23,24] A semimicro combination electrode (Sargent, Model 30070-10) was connected to a pH meter (either a Radiometer Model TTTlC regulator unit or a Corning Model 12 Research meter), the output signal from which was attenuated so that 100 mV equalled one pH unit full scale, and was applied to a Sargent Model SR recorder. All components were carefully grounded and the electrode and reaction vessel enclosed in a Faraday cage to minimize stray potential interference. A small potential applied through a variable potentiometer allowed any pH reading to be brought onto scale. A range switch on the recorder allowed full-scale readings of 0.01 to 1.0 pH unit. The 10–25-ml reaction vessel was water jacketed and thermostated at 25°. Gilson micrometer burets were used to add acid, base, substrate, or inhibitor to the reaction vessel *via* Teflon needles fitted with Luer joints. The reaction solution was stirred as rapidly as possible (without causing foaming or cavitation) with a 10-mm Teflon-covered magnetic stir bar. Water-saturated, CO_2-free nitrogen was swept through the tightly covered reaction vessel to exclude CO_2 from the air.

Typically, the electronic components of the apparatus were warmed up at least 1 hr prior to use, after which the electrode was calibrated with several standard buffers in the pH range of interest.[25] Then 10 ml of enzyme solution was pipetted into the reaction vessel, and allowed to equilibrate at 25° with the nitrogen sweep, after which standard base was used to raise the pH to the proper value. At each pH of interest three experiments were carried out. First, pure inhibitor was added to a solution of inactive enzyme, either zymogen or diisopropylphosphoryl-α-chymotrypsin. Second, an equimolar amount of standard base was added to 0.1 m*M* active enzyme, either α- or δ-chymotrypsin. Third, pure inhibitor was added to a solution of the chosen active enzyme. The first experiment provides a blank to check for impurities in the inhibitor solution. The second experiment artificially produces a pH change equal to that expected upon absorption of one proton per mole of enzyme. Comparison of the pH change observed in the third experiment with that of the second leads to a calculation of *n*, the fraction of a proton absorbed per mole of enzyme.

A spectrophotometric technique with pH-indicator dyes was developed in addition to the pH-recorder technique. The maximal absorbances of thymolsulfonphthalein (thymol blue) and phenolphthalein anions occur at 598 and 553 mμ, respectively. Titration curves were then determined for each dye, using these wavelengths, so that observed absorbances could be converted into pH readings. The region of pH 8.4–9.4 was considered useful for thymol blue, and pH 8.9–9.9 for phenolphthalein. At concentrations of 2.8×10^{-5} *M* thymol blue or 5.0×10^{-5} *M* phenolphthalein, specific substrate assays[17] indicated that no dye binding or inhibition occurred at the active site of the enzyme.

Typically, 3.0 ml of unbuffered enzyme solution plus dye was thermostated in a Pyrex cuvette in the Cary 14 cell compartment at 25°. The pH was then adjusted with standard base, and at each pH of interest, the three separate experiments outlined above were carried out, leading to a calculation of *n*.

Contrary to observations published elsewhere,[13] for small pH changes, the enzyme exerts a definite buffering effect. In the region of pH 6 to 10, also, a pronounced downward pH drift was observed with the active enzymes, but not with either inactive enzyme species. The pH dependence of the rate of drift approximately parallels the rate of spontaneous denaturation of the enzyme, attributed to autolysis and unfolding at these pH's.[26] Addition of inhibitors greatly slowed down this pH drift, as might be expected, except above pH 9.5 where the enzyme is much less stable. Acetonitrile also had the effect of accelerating the rate of drift at high pH and in high concentrations.

Results

Chymotrypsinogen A. The extent of benzyl alcohol–chymotrypsinogen binding, as determined by equilibrium dialysis experiments at pH 7.8, is presented in Table I. Without exception, the inhibitor concentration at the conclusion of the experiment was higher in the enzyme solution than in the surrounding buffer regardless of whether inhibitor was introduced initially inside or outside the dialysis bag. Enzyme precipitation prevented determination of binding data at 4–5 m*M*

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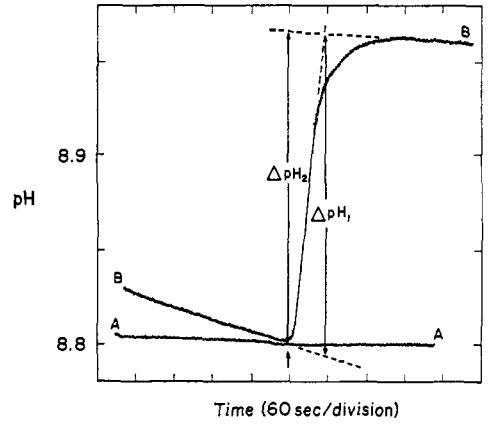

Figure 1. A typical trace for the recording pH meter apparatus, observed pH against time, involving 10^{-4} M chymotrypsinogen A (curve A) and 10^{-4} M α-chymotrypsin (curve B), 0.1 M KCl, upon addition of benzyl alcohol (K_i = 10 mM, pH 7.9) to give a final concentration of 200 mM; 25°. The "observed ΔpH" was calculated using the graphically determined values and

$$\Delta pH_{obsd} = (\Delta pH_1 + \Delta pH_2)/2$$

from which could be calculated a value for n (see text).

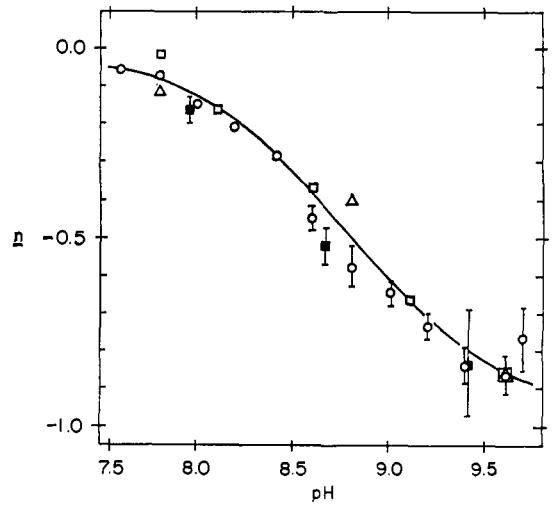

Figure 2. The pH dependence of proton absorption, n, by 10^{-4} M α-chymotrypsin upon binding of inhibitors. pH-recorder data: benzyl alcohol (○), acetonitrile (△), N-acetyl-D-tryptophanamide (□), $[I_0]$ always five times $>K_i$ at each pH; 25°. pH-indicator dye technique with thymol blue and phenolphthalein: α-chymotrypsin, 10^{-4} M, and benzyl alcohol, ■. The solid line is calculated from eq 5, assuming pK_a(app) = 8.8.

inhibitor. Analysis of these data, *i.e.*, a plot of r against $r/[I]$, according to the equation

$$r = N - K_m(r/[I]) \quad (1)$$

where r is the moles of inhibitor I bound per mole of enzyme, and N is the number of binding sites per protein molecule, gives values of the binding constant K_m = 11 mM and N = 1. These results confirm those of previous studies:[27] neutral inhibitors or substrates bind to a single site on the zymogen nearly as well as to α-chymotrypsin.

Table I. The Binding of Benzyl Alcohol to Chymotrypsinogen A, as Determined by Equilibrium Dialysis, pH 7.8,[a] 25°, $E_o = 1.0 \times 10^{-3}$ M

$[I_{free}]$, mM	r, mM[b]	$r/[I_{free}]$, M^{-1}
4		
12.8	0.55	4.30
10.9	0.60	5.35
21.5	0.80	3.65
22.0	0.70	3.13

[a] Borate buffer, 0.05 M, μ = 0.10 M. [b] r = moles of I bound per mole of protein; I = benzyl alcohol.

Using the pH-recorder apparatus, no noticeable change in the state of protonation of chymotrypsinogen could be observed at any pH from 7.0 to 9.6 upon addition of saturating amounts of benzyl alcohol (Figure 1, curve A). The phosphorylated (DIP) enzyme gave the same result, in contrast to that obtained with active enzyme forms, presented below.

α-Chymotrypsin. Again using the pH recorder apparatus, above pH 7 the addition of saturating amounts of benzyl alcohol to α-chymotrypsin in unbuffered solution resulted in an immediate pH rise to a maximal value (Figure 1, curve B). The initial pH drift, not observed with the zymogen (curve A), is attributed to autolysis and is greatly diminished in the presence of inhibitor. Thus, binding alone causes proton absorption by α-chymotrypsin.

Next, three inhibitors were added separately in turn to α-chymotrypsin in saturating concentrations, and each was observed to cause proton uptake. In order of increasing binding (K_i^{-1}) specificity these were: acetonitrile, benzyl alcohol, and N-acetyltryptophanamide (the D and L isomers separately). Benzyl alcohol, which mimics the side-chain group of a specific amino acid inhibitor, was the most widely used due to its high solubility/K_i ratio. The calculated values of n for each inhibitor are presented in Figure 2. No proton absorption could be observed below pH 7.0, although the pH recorder was fully sensitive enough to detect even small pK_a shifts had they occurred.

To prove that the observed proton uptake upon addition of acetonitrile (up to 10% by volume) was not an apparent phenomenon resulting from altered solution conditions, such as dielectric constant, the following control experiment was carried out: at pH 8.80 benzyl alcohol (200 mM) was added, causing uptake of 0.5 proton per mole of enzyme, after which a saturating amount of acetonitrile was added. No further change in pH resulted from this second addition. The experiment was performed in reverse with the same result. The control reaction with zymogen or DIP-α-chymotrypsin and acetonitrile indicated no pK_a shift with any inhibitor.

The data of Figure 2 clearly indicate that a single proton is absorbed in the binding process of chymotrypsin at alkaline pH, and the pK_a = 8.8 strongly suggests that proton uptake and binding inhibition are linked processes. To prove this correlation in a more quantitative fashion, two complementary experiments were carried out at a series of pH's from 7.8 to 9.6 with each inhibitor.

(a) The observed n value or "proton signal" was determined as a function of inhibitor concentration, from which a dissociation constant, K_d, was calculated,

(27) F. Vaslow and D. G. Doherty, *J. Amer. Chem. Soc.*, 75, 928 (1953).

using reciprocal plots of n and [I] as with v and [S] kinetic data (Figure 3).

(b) With methyl N-acetyl-L-tryptophanate as substrate, competitive inhibition experiments were performed, from which a K_i value was obtained. Table II

Table II. The pH Dependences of Proton Absorption and Inhibitor Binding Constants (K_d and K_i) for α-Chymotrypsin, 25°[a]

pH	n_{max} (obsd)	K_d, mM[b]	K_i, mM[c]
		Benzyl Alcohol	
7.8	0.10	17 ± 7	9.8 ± 2
9.0	0.55	17 ± 2	20.5 ± 6
9.7	0.87	50 ± 2	51.0 ± 10
		Acetonitrile	
7.8	0.10	750	*d*
9.0	0.60	1200	*d*
9.7	0.90	*d*	*d*

[a] The value n_{max} was determined using saturating amounts of inhibitor. [b] Determined by proton absorption, n, as a function of [I]. [c] Determined by competitive inhibition experiments, using methyl N-acetyl-L-tryptophanate and an inhibitor concentration at least twofold greater than the observed K_i, buffer = 0.05 M, μ = 0.1 M. [d] Rapid disruption of enzyme activity occurred in these experiments, due to high solvent concentration.

presents the experimental values of n_{max}, K_d, and K_i for benzyl alcohol and acetonitrile with α-chymotrypsin. The dissociation and inhibition constants agree quite well. The pH dependences of K_d, K_i, and n_{max} are predicted accurately by the equations derived for the theoretical scheme presented in the Discussion section: proton uptake and binding inhibition exactly parallel each other in a reciprocal fashion.

The experiments with N-*trans*-indoleacryloylimidazole described by Bernhard[13] for α-chymotrypsin were repeated and the calculated n values agreed exactly. The pH changes due to acylation and deacylation were carefully observed: at pH 8.8 acylation resulted in uptake of 0.5 proton per mole of enzyme, and deacylation liberated 1.5 protons per mole of enzyme, 1.0 due to the acid product liberated and 0.5 due to reappearance of the proton absorbed upon acylation. The calculated deacylation rate, 1.23×10^{-2} sec^{-1} at pH 8.8, agrees within 10% of that observed spectrophotometrically.

Changes in the enzyme's state of protonation at high pH were also observed *via* a spectrophotometric technique with pH-indicator dyes. Once again, the zymogen was used in a control reaction. The pH dependence of n calculated for saturating concentrations of benzyl alcohol is in excellent agreement with the results obtained with the pH recorder, as shown in Figure 2.

δ-Chymotrypsin. Since the main chemical difference between the zymogen and the δ form is the presence of two new chain terminals formed in the activation process, the pH dependence of the proton-absorption process should identify the group exhibiting a pK_a shift upon inhibitor binding. With the proper controls, proton absorption was observed at a series of pH's upon addition of benzyl alcohol to δ-chymotrypsin. The apparent pK_a of the proton uptake process is 8.8, strikingly identical with that observed for the binding inhibition phenomenon at alkaline pH.

Because the acylation of α-chymotrypsin with N-*trans*-indoleacryloylimidazole was reported to result in proton absorption,[13] it was shown that δ-chymotrypsin

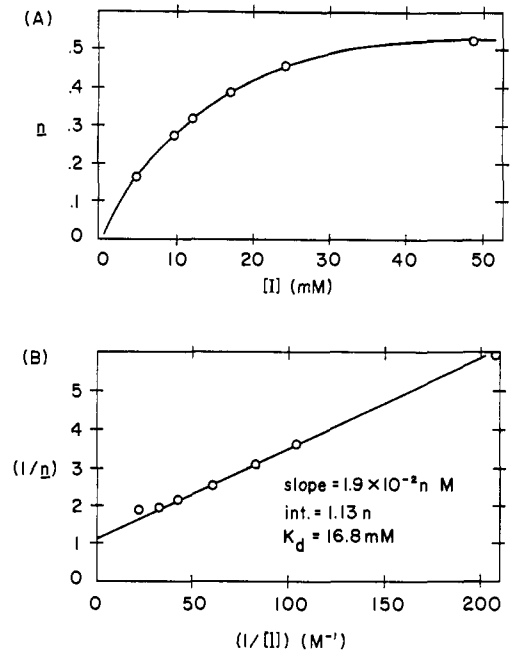

Figure 3. The dependence of n, the number of protons absorbed per mole of enzyme, the "proton signal," upon the added benzyl alcohol concentration, pH 9.0, 0.1 M KCl, 25°, using 10^{-4} M α-chymotrypsin. Curve A: n against [I]; curve B: n^{-1} against $[I]^{-1}$, with the experimental value of the dissociation constant, K_d, for the enzyme-inhibitor complex. The solid lines are the estimated best fit to the data, but can also be calculated from eq 5.

also exhibits this effect. Thus, in the over-all acylation process (k_2/K_s) proton uptake occurs solely in the binding step (K_s) preceding acylation (k_2). No further proton uptake occurs in the k_2 step, since it is all accounted for in the binding step. Another pK_a shift in the region of pH 6–7 has been observed and discussed by Bernhard[13] and by Glick,[28] presumably involving the imidazole of histidine-57.

Discussion

The above results primarily imply that chymotrypsin's binding ability and the protonation of a single group near the active site are intimately related. All earlier suggestions of this relationship were based on a very approximate identity of the pK_a's of the separately observed processes.[4-6,13] With such direct proof now in hand, especially the data of Figure 3, these other phenomena (*e.g.*, spectral changes) can be considered related to and part of the high pH mechanism of α-chymotrypsin.

The simplest model scheme to represent such coupled equilibria is shown in eq 2, which, for the moment, ignores any ionizations other than the pK_a = 8.8. Sub-

$$\begin{array}{ccc} E & \underset{H^+}{\overset{K_a}{\rightleftharpoons}} & EH^+ \\ & & S \updownarrow K_s \\ & & EH^+S \end{array} \qquad (2)$$

strate binds preferentially to the protonated enzyme form EH^+, but not to the deprotonated form E; at a given pH the saturation of enzyme with substrate S converts all E into EH^+, and a proton is absorbed for each mole of E thus converted. The *apparent* binding constant increases with pH since more S is required to

(28) D. M. Glick, *Biochemistry*, 7, 3391 (1968).

attain saturation and force the equilibrium in favor of EH^+. The mathematical expressions for $K_m(app)$ and n as functions of pH and [S] derived from the model scheme of eq 2 predict curves which agree with the data presented in Figures 2 and 3.

The model of eq 2 implies nothing more complicated than substrate binding and proton uptake occurring at the same enzyme surface site. Why should a neutral hydrophobic molecule bind preferentially to a charged (protonated) site rather than to a neutral one? Recent evidence suggests[3-9] that above pH 7 the active site of chymotrypsin can assume two conformations. If one defines that at high pH E* forms of chymotrypsin which *cannot* bind substrate predominate, whereas at low pH E forms which *can* bind substrate predominate, and that the pK_a's for E and E* are quite different, then protonation controls the conversion of E to E* and is linked directly to substrate binding, as shown in eq 3. To in-

$$\begin{array}{ccccc} E^*H^+ & \overset{K_{TH}}{\rightleftharpoons} & EH^+ & \overset{K_S}{\rightleftharpoons} & EH^+S \\ \Big\updownarrow K_A^* & & \Big\updownarrow K_A & & \Big\updownarrow K_A' \\ E^* & \underset{K_T}{\rightleftharpoons} & E & \underset{K_S'}{\rightleftharpoons} & ES \end{array} \qquad (3)$$

clude the binding of substrate with E* forms of the enzyme needlessly complicates the derivations, since one cannot accurately estimate or determine the magnitude of enough microscopic constants in such a scheme to lead to simplified expressions for $K_m(app)$ or n as functions of pH or [S]. Only if one considers nonspecific substrate binding at high pH is inclusion of S-E* binding justifiable.[3,5]

Since E* dominates at high pH, $K_T = [E]/[E^*] \ll 1$; and since EH^+ dominates at low pH, $K_{TH} = [EH^+]/[E^*H^+] \gg 1$. Assuming that protonation affects substrate binding only *via* a transformation from E to E*, it is reasonable to assume that $K_A = K_A'$ and that $K_s = K_s'$. With these simplifying assumptions, the equations one derives from the model of eq 3 for $K_m(app)$ and n are

$$K_m(app)^{-1} = K_s^{-1}\left(\frac{1}{1 + \frac{K_A}{K_T[H^+]}}\right) \qquad (4)$$

$$n = \left(\frac{1}{1 + \frac{K_A}{K_T[H^+]}}\right) - \left(\frac{1 + \frac{[S]}{K_s}}{1 + \frac{[S]}{K_s} + \frac{K_A}{K_T[H^+]}}\right) \qquad (5)$$

The equations derived from eq 2 are the same, except that K_A/K_T in eq 4 and 5 is replaced by K_a. Table III presents calculated values of $K_m(app)$ and n from eq 4 and 5 as functions of pH. These values were plotted as a solid line in Figure 2 and are seen to agree with the experimental data quite well. Unless other critical changes in the enzyme can be observed near pH 9, this model should be considered adequate to predict all behavior of α-chymotrypsin at high pH with specific substrates or inhibitors.

Two recent studies bring into apparent contention the magnitude of the pK_a shift near pH 9 upon binding, and therefore deserve comment at this time. Glick[28] reports a shift of only 0.3–0.6 unit for α-chymotrypsin, and Odell, *et al.*,[29] report shifts of *ca.* 0.6 and ≥ 1.0 for δ-chymotrypsin and α-chymotrypsin, respectively.

(29) C. Odell, G. Cerlinski, and G. P. Hess, *Science*, 161, 274 (1968).

Table III. The Theoretical pH Dependence of $K_m(app)$ and n_{max} ($[S]/K_s \gg 1$), Calculated from Eq 4 and 5, Assuming $p(K_A/K_T) = 8.80$ in the Model of Eq 3

	pH	$K_m(app)$	K_m^{-1}	n_{max}	$n_{max} + 1$
	6.8	1.01	0.99	−0.009	0.99
	7.8	1.10	0.91	−0.091	0.91
	8.1	1.20	0.83	−0.167	0.83
	8.4	1.39	0.72	−0.286	0.71
	8.6	1.57	0.64	−0.369	0.63
$pK =$	8.8	2.00	0.50	−0.500	0.50
	9.1	3.00	0.33	−0.667	0.33
	9.5	6.00	0.17	−0.833	0.17
	9.8	11.08	0.09	−0.909	0.09
	10.1	20.98	0.05	−0.952	0.05
	10.8	100.1	0.01	−0.990	0.01

The data of Figure 2 above, however, agree with those of Bernhard[13] and of Garel, *et al.*,[30] which imply that the pK_6 must be shifted at least 1.5 units, or from 8.8 to 10.3 or higher. In all these latter studies, substrate or inhibitor saturation $\geq 75\%$ was attained. A shift to $pK \geq 10.3$ is also implied by previously published high pH binding[3-5,31] and difference titration[5] data. It is suggested that smaller pK_a shifts were observed because: (a) background effects above pH 8.5 were not controlled carefully enough,[28] (b) only partially specific inhibitors were employed,[28] and (c) data at only 30–50% saturation were extrapolated to estimate maximum proton signal at 100% saturation.[28,29] Without proton signals above $n = 0.2$–0.3, such extrapolations lead to underestimation of n_{max}.[32] Further, preliminary observations in this laboratory indicate significant differences in the binding abilities of δ- and α-chymotrypsins.

The α-ammonium group of isoleucine-16 at the B chain N terminus has been clearly identified as the pK 8.8 group which controls binding.[5] Since the intrinsic pK_a of an N-terminus α-ammonium group is *ca.* 7.8, the *apparent* pK_a of 8.8 does not serve to identify the isoleucine group, being fully one pH unit too high. This *apparent* pK_a is a combination of two terms or processes, shown in eq 4 and 5 as $p(K_A/K_T)$. Since $p(K_A) \geq 10.3$ for all EH^+S forms, the K_T^{-1} term must be ≥ 30. Upon binding of substrate, all E* enzyme forms convert to EH^+S forms, accompanied by proton uptake. To maintain a $p(K_A) \geq 10.3$, the isoleucine's ammonium ion must exist in an extremely polar region.[33] The ionic bond now known to exist between the isoleucine-16 ammonium group and the carboxylate side-chain group of aspartate-194 should adequately provide such an environment.[34]

All present data indicate that the active site conformation change of chymotrypsin at high pH alters its binding or catalytic properties only in a negative fashion. Based on recently published data about the structure of chymotrypsin,[34] one may speculate concerning the mechanism of the energetically favorable conversion of E to E* in the K_T step of eq 3. The first possibility is that the N terminus of the B chain in the deprotonated form competes favorably with substrate for the apolar

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binding site, and that protonation of the α-amino group of isoleucine-16 makes the chain terminus a less effective competitor, especially since it is tied back in the ionic bond with the side-chain carboxylate of aspartate-194. The second possibility is that protonation and formation of the ionic bond stabilize an active site conformation which is otherwise energetically unfavorable, and that deprotonation results in partial collapse of the site's critically defined structure. This latter suggestion seems more likely, based on data with nonspecific substrates of chymotrypsin. With *p*-nitrophenyl acetate or methyl hippurate[35] at high pH, catalysis rather than binding is altered;[4] that is, one observes a pH-dependent intramolecular noncompetitive inhibition phenomenon. From this one concludes that the conformational change alters the position of serine-195 relative to histidine-57. Hence, upon breaking the isoleucine-16–aspartate-194 ionic bond the polypeptide loop containing serine-195 and aspartate-194 collapses into the apolar site. Such collapse would prevent binding of molecules of a particular size, hydrophobic character, or specificity for part of the active site, but not those without such characteristics.

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Maxwell Relations for Thermodynamic Quantities of Biochemical Reactions

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Abstract: Since equilibrium calculations for biochemical reactions such as reaction 1, ATP[1] + $H_2O \rightleftharpoons$ ADP + P_i, are generally carried out with expressions of the type K_{obsd} = (ADP)(P_i)/(ATP), where the concentrations include all species, the various thermodynamic quantities are all functions of pH and concentrations of any cations present which form complexes with reactants or products as well as temperature, pressure, and electrolyte medium. In this paper the only complexing cation considered to be present is Mg^{2+}, so that the thermodynamic quantities are considered to be functions of only temperature, pH, and pMg[2] at constant ionic strength and 1 atm. The Maxwell relations give relations between rates of change of thermodynamic quantities with respect to different independent variables. For example, it is shown that the rate of change of the heat of reaction with pH is proportional to the rate of change of the production, n_H, of H^+ with temperature. The rate of change of the heat of reaction with pMg is proportional to the rate of change of the production, n_{Mg}, of Mg^{2+} with temperature. Equations are also derived for $\partial\Delta S^\circ_{obsd}/\partial pH$, $\partial\Delta S^\circ_{obsd}/\partial pMg$, $\partial\Delta C^\circ_{p,obsd}/\partial pH$, and $\partial\Delta C^\circ_{p,obsd}/\partial pMg$ in terms of n_H, n_{Mg}, $\partial n_H/\partial T$, $\partial n_{Mg}/\partial T$, $\partial^2 n_H/\partial T^2$, and $\partial^2 n_{Mg}/\partial T^2$. Contour plots are given for $\partial\Delta G^\circ_{obsd}/\partial pH$, $\partial\Delta G^\circ_{obsd}/pMg$, $\partial\Delta H^\circ_{obsd}/\partial pH$, $\partial\Delta H^\circ_{obsd}/\partial pMg$, $T(\partial\Delta S^\circ_{obsd}/\partial pH)$, and $T(\partial\Delta S^\circ_{obsd}/\partial pMg)$ for reaction 1 which present the dependence on pH and pMg at 25° and 0.2 ionic strength in the range pH 4–10 and pMg 1–7. The equation is derived for the change in heat capacity, $\Delta C^\circ_{p,obsd}$, for reaction 1 and estimates of this quantity are also presented for the range pH 4–10 and pMg 1–7. In order to estimate $\Delta C^\circ_{p,obsd}$ it has been necessary to assume values of ΔC°_p for the dissociations of ATP and ADP on the basis of analogy with structurally related compounds.

The observed equilibrium constant, K_{obsd}, for the hydrolysis of ATP written in terms of the total

$$ATP + H_2O \rightleftharpoons ADP + P_i \quad (1)$$

$$K_{obsd} = (ADP)(P_i)/(ATP) \quad (2)$$

concentrations of reactants and products is a function of pH[3–5] and metal ion concentrations.[6–10] With data on the observed equilibrium constant at one pH and free metal ion concentration and with dissociation constants for the weak acids and complexes it is possible to calculate the standard Gibbs free energy of hydrolysis at various pH values and metal ion concentrations (eq 3). This is the change in Gibbs free energy when the

$$\Delta G^\circ_{obsd} = -RT \ln K_{obsd} \quad (3)$$

reactants designated in the equilibrium constant expression, each in the standard state of (hypothetical) 1 *M* solution, are converted to the products designated in the equilibrium constant expression, each in the standard state of (hypothetical) 1 *M* solution, all at the designated temperature in an electrolyte solution of the specified ionic strength having the designated pH and free metal ion concentration. With data on the heat of hydrolysis of ATP and the enthalpies of ionization and complex dissociation of the acidic forms and magnesium complexes of ATP, ADP, and P_i, the values of ΔH°_{obsd} and ΔS°_{obsd} for the over-all reaction may be obtained[11]

(1) ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, orthophosphate.

(2) pMg is defined for Mg^{2+} in a similar way to pH for H^+ and is assumed to be obtained by use of a reversible divalent cation electrode.

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