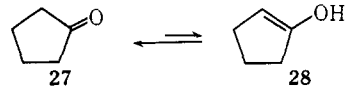
The distinctions between acylaminoglycocyamidines of type I (exocyclic C=N bond) and type II (endocyclic C=N bond) are much less clear cut. The similarities of both the ultraviolet absorption maxima and pK_a values for the two types do not offer any source of differentiation. It should be noted, however, that the molar extinction coefficients for the two type I compounds were observed to be greater than those for the rest of the acylaminoglycocyamidines. Similarly, the two type I compounds were less stable toward hydrolysis at pH 12 and showed more rapid deuterium exchange rates for their ring methylene hydrogens than the other acylaminoglycocyamidines. Finally, type I compound **4b** has an *N*-methyl group (presumably the C=N—CH_3) which showed an unusually low-field chemical shift (δ 3.10) in its nmr spectrum, whereas type II compound **5b** has no *N*-methyl peak below 2.95 (Table I), which is consistent with its assigned tautomeric preference.

Our assigned preference of endocyclic C=N bonds over exocyclic C=N bonds for the glycocyamidines is in agreement with the known preference of endocyclic C=C bonds over exocyclic C=C bonds in five-membered ring systems. For example, the equilibrium between methylenecyclopentane (**25**) and 1-methyl-1-cyclopentene (**26**) has been shown by Cope, *et al.*,[30] to lie far to the side of the endocyclic C=C bond system, *i.e.*

25 (0.09%) **26** (99.91%)

The preponderance of **26** over **25** had earlier been asserted by Brown.[31] On the other hand, as Brown pointed out,[31] there is a large preference for an exocyclic C=O bond (keto) over an endocyclic C=C bond



27 **28**

(enol) in cyclopentanone. We believe, however, that the equilibrium between the tautomers of the acylaminoglycocyamidines is more closely analogous to the equilibrium of **25** and **26** than to the equilibrium of **27** and **28** because the double bond is of the same type, C=N, for both the endocyclic and exocyclic isomers, whereas this is not the case for the equilibrium of **27** and **28**.

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(30) A. C. Cope, D. Ambros, C. Ciganek, C. F. Howell, and Z. Jacura, *J. Amer. Chem. Soc.*, **82**, 1750 (1960).

(31) H. C. Brown, *J. Org. Chem.*, **22**, 439 (1957).

Substrate-Induced p*K* Perturbations with Chymotrypsin[1]

James L. Marini and Michael Caplow*

Contribution from the Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514. *Received December 10, 1970*

Abstract: Proflavin binding to chymotrypsin is pH dependent in the near-neutral pH range and the dissociation constants for the dye with the enzyme conjugate acid and base are 12.19×10^{-5} and 3.25×10^{-5} *M*, respectively. The p*K* of the proflavin–enzyme complex is 5.60 and the free enzyme has a p*K* of 6.18. Dye displacement has been used to study complexation of substituted benzamides and furoylamide with the enzyme to determine whether equivalent p*K* perturbations are produced on noncovalent binding of acyl groups as is seen with the corresponding groups covalently linked to the enzyme in isolable acyl enzymes. There is no p*K* perturbation produced by binding of benzamide and furoylamide; *p*-nitrobenzamide induces a small p*K* perturbation. Covalent binding of these acyl functions to the enzyme is required for p*K* perturbation. The dissociation constants for benzamide, *p*-nitrobenzamide, and furoylamide are 3.06 m*M*, 1.34 m*M*, and 13.86 m*M*, respectively, at pH 7.7, 25°. The dissociation constant for *p*-methoxybenzamide is approximately 1.22 m*M* under these conditions. It is proposed that acylation of the enzyme increases the p*K* of the active-site histidine residue by disruption of the hydrogen bond between His-57 and Ser-195. Hydrogen bonding between the histidine residue and the acyl linkage of the acyl enzyme results in further increases in the p*K*. Hydrogen bonding with the acyl linkage requires covalent bonding of the acyl group to the enzyme.

Studies of the acylation of chymotrypsin with anilides of a specific substrate—acetyltryptophan derivatives—and deacylation of isolable acyl enzymes formed from nonspecific substrates have shown that the pH dependence for activity depends on the electronic properties of the acyl function; electron withdrawal by either the acyl or aniline moiety results in a lowered apparent p*K* for activity.[2] Proton dissociation from the critical histidine residue required for activity is, therefore, coupled with some other equilibrium process. The reversible formation of a tetrahedral intermediate has been suggested to be responsible for p*K* perturbation with anilides;[2a] evidence against this process underlying the p*K* perturbation with acyl enzymes has been

(1) Supported by grants from the National Institutes of Health (DE 02761 and DE 03246).

(2) (a) M. Caplow, *J. Amer. Chem. Soc.*, **91**, 3639 (1969); (b) Vishnu and M. Caplow, *ibid.*, **91**, 6754 (1969); (c) S. A. Bernhard, E. Hershberger, and J. Keizer, *Biochemistry*, **5**, 4120 (1966).

described.[2b] For the latter compounds, some form of weak interaction not involving covalent modification of the acyl function is presumed to be responsible for the results.

To determine whether the enzyme pK may be perturbed without covalent bond formation to the active-site serine residue or to the acyl function we have carried out a study of complexation of chymotrypsin with a series of amides with similar steric properties and varying electronic substituents. These compounds—para-substituted benzamides and furoylamide—do not acylate the enzyme and are not hydrolyzed. Since the acyl linkage remains intact, if equivalent pK perturbations are induced by amide binding as observed with the corresponding acyl chymotrypsins, it would not be necessary to involve reversible covalent modification of the acyl linkage to account for the pK perturbations seen with the latter systems.

It has been found that binding of amides does not induce pK perturbations equivalent to those observed with the corresponding acyl enzymes; pK perturbation occurs on binding *p*-nitrobenzamide but does not occur with furoylamide, benzamide, and *p*-methoxybenzamide. Covalent bonding of the acyl function to the enzyme is required for the pK perturbation seen in kinetic studies.

Experimental Section

Visible and ultraviolet spectra were obtained with a Zeiss PMQ II spectrophotometer. Slit widths for most studies in the uv were generally 0.07 mm, and in the visible, from 0.018 to 0.030 mm. Spectra were obtained using matched 1- and 5-cm cells, and 1-cm cells with 0.8- and 0.9-mm spacers (for 0.2- and 0.1-cm path lengths). Cell matching was checked for all cells on every dye binding run. Cells were thermostated to 25 ± 0.5° during dye binding runs by means of water circulating through the cell compartment housing.

pH measurements were made on an Orion Model 801 meter with a Sargent S-30070-10 glass electrode at 25.0 ± 0.1°. All melting points are corrected. α-Chymotrypsin, three-times recrystallized, was obtained from Worthington (lots CDI 7KE and CDI 8LK); proflavin sulfate was supplied by Mann Research Laboratories. All chemicals and solvents used were reagent grade unless otherwise specified.

Purification of Proflavin. Commercial proflavin sulfate, $(C_{13}H_{11}N_3)_2H_2SO_4 \cdot 2H_2O$, was received as a red powder. Three recrystallizations from glass-distilled water gave red plates, mp 269° dec (sealed tube). The recrystallized product was dried to constant weight at 0.5 mm and room temperature over Drierite (*ca.* 18 hr), and stock solutions of 2×10^{-3} M dye were prepared from the dried proflavin. Dye concentrations were calculated on the basis of the molecular weight of the dihydrate, 552.62, since drying at room temperature, although necessary because of the dye's hygroscopicity, was not expected to result in loss of water of crystallization. All manipulations with the dye or dye solutions were carried out with minimum exposure to light.

A master stock solution of dye was made up in freshly boiled glass-distilled water and stored in a red glass volumetric flask out of light. New master stock solutions were made at about 3-month intervals, although a check after 3 months on the first stock solution showed no significant change in E_{max} of the dye. However, some black, insoluble residue was observed when an aliquot was removed from the bottom of the stock solution after about 3 months' standing.

Proflavin solutions for dye-binding studies were made up by diluting the proflavin stock solution with the appropriate buffer; the final dye concentration was calculated from the *weight* of the master stock solution used, assuming a density of unity, and from the nominal volume of the volumetric flask employed. The extinction coefficients of these dye solutions were measured at 465 nm in 5-cm cells before every dye-binding experiment to ensure that no significant decomposition of the dye stock solutions or dye–buffer solutions had occurred. Only one of dozens of dye–buffer solutions showed a significant change in absorbance at 465 nm (a decrease), and only on at least 2 weeks standing.

Buffer Solutions. Buffer solutions were made with sufficient concentration for ionic strength 0.10 ± 0.01; glass-distilled water was used throughout.

Buffer densities were determined in triplicate measurements at 23–25° and were in the range 1.004–1.017. Reproducibility to 0.1% was demonstrated by density determinations on two different batches of pH 4.6 acetate buffer.

Dye-Binding Procedure. The crystalline enzyme, stored at 2°, was allowed to warm to room temperature, after which a weight of enzyme required to give a stock solution of $1–1.1 \times 10^{-3}$ M was weighed into a flask to which was then added the required volume of buffer, previously cooled to 2°. The mixture was then refrigerated for 15–45 min for solution to occur with a minimum of autolysis. Removal of a trace of insoluble material was accomplished either by centrifugation for 20–40 min at 28,000 rpm in the no. 40 head of a Spinco centrifuge, or by filtration through a LSW PO1300 Millipore filter.

A 1-ml aliquot of this enzyme solution was weighed into a tared 10-ml volumetric flask which was then filled with buffer and reweighed. The absorbance of the resulting *ca.* 10:1 diluted enzyme solution was read at 282 nm in a 0.1-cm cell *vs.* buffer, and the protein concentrations were calculated from the extinction coefficient, 5×10^4 O.D. M^{-1} cm^{-1}.[3] The concentration of the *ca.* 1×10^{-3} M enzyme stock solution could then be calculated in moles/liter and in moles/gram, given the density of the buffer used. The enzyme stock solution was stored at 2° when not in use.

A 1-ml portion of the proflavin stock solution (*ca.* 4×10^{-5} M in the appropriate buffer) was then diluted 10:1 as for the enzyme, and the resulting solution's absorbance determined at 465 nm in a 5-cm cell *vs.* buffer. The reference cell, when filled with the remaining 4×10^{-6} M dye solution, served as the blank for dye-binding runs. The ratio of the mass of dye stock solution to the total mass of reference solution, around 0.1, was calculated to five decimal places. During manipulations, solutions containing dye were stored out of direct light except when being filled or weighed.

Dye–enzyme solutions were prepared as follows. Either one at a time or in batches of up to six at a time, 0.5 ml of the 4×10^{-5} M dye stock was weighed into a tared 5-ml volumetric flask. The required weight of enzyme plus buffer solutions needed to give the same dye–total weight ratio as the blank was then added. After the total weight was taken, the flask was shaken, wrapped in aluminum foil, and suspended in a 25.0 ± 0.1° bath. This entire procedure for six–eight samples required from 45 to 60 min.

The absorbances of the dye–enzyme solutions were measured after the last sample was prepared. At least ten absorbance readings were made on each sample (requiring less than 2 min) in order to check for drift in the data and other anomalies. After completing the O.D. readings an aliquot of the sample was used for pH measurements; this was done within *ca.* 2 min. The entire experimental procedure, including sample preparation, required about 4 hr.

The data were worked up as follows. The observed absorbance was corrected for cell mismatching, absorbance by the enzyme (using an extinction coefficient equal to $1.1 \times 10^1 \times$ O.D. mol^{-1} cm^{-1} at 465 nm), and, infrequently, for mismatching of the dye concentration in the sample and reference solutions. The dissociation constant for the dye (K_D) and the difference in the extinction coefficients of the dye and enzyme–dye complex (ΔE_m) were obtained from a computer-calculated least-squares fit of a plot of difference-absorbance *vs.* enzyme concentration.[4] In order to determine whether enzyme autolysis or denaturation occurred during the rather long time required for sample preparation several runs were carried out (at pH 5.4) which were analogous to the described procedure, except that weighings were omitted and concentrations were adjusted using volumetric procedures. Each dye–enzyme sample was run independently so that, with a 5-min equilibration at 25°, the enzyme was at temperatures greater than 2° for no more than 10 min. The results so obtained were in excellent agreement with those obtained with the gravimetric procedure, although the data showed more scatter with the more rapid method; we attribute this principally to mismatching of the dye concentrations in the sample and reference cells. Further evidence on this point comes from our observation that there was no discern-

(3) K. G. Brandt, A. Himoe, and G. P. Hess, *J. Biol. Chem.*, 242, 3973 (1967).

(4) K. R. Hanson, R. Ling, and E. Havir, *Biochem. Biophys. Res. Commun.*, 29, 194 (1967).

ible trend away from a theoretical Michaelis–Menten equilibrium with points taken in the later stage of experiments.

Inhibitor Experiments. Enzymatic and nonenzymatic hydrolysis of the amides was assumed to be negligible. Determination of the amide–enzyme dissociation constants, K_s, was made by a slight modification of the gravimetric dye-binding procedure described above.

Benzamide and *p*-methoxybenzamide were recrystallized three times from glass-distilled water, giving samples of mp 127.5–128.3° and 167–167.8°, respectively. *p*-Nitrobenzamide was recrystallized once from *ca.* 50:50 v/v ethanol–water, yielding fine needles with mp 199.2–200.3°. 2-Furoylamide was synthesized from furoyl chloride by treatment with ammonia in ether, and the crude amide (mp 140–143°) was crystallized twice from 30% dioxane in ether giving a light yellow product, mp 141.5–142.5°. Samples of 2-furoylamide used in the inhibitor studies were further purified by one or two sublimations at 90–110° (0.5 mm), giving well-formed, white crystals, mp 141.5–142.5° (lit. 141–142°, 142–143°[5]).

The determination of K_s for each amide was carried out as for the determination of K_D except that stock solutions of the amides in the appropriate buffer were used instead of or in addition to the buffer. The densities of all amide solutions were taken equal to the buffer density. This assumption was checked on a 1.47×10^{-2} *M* solution of *p*-methoxybenzamide, pH 7.7, the density of which was found to be 1.003 ± 0.004 g/ml (buffer, 1.006 ± 0.004 g/ml). Concentrations of the amides, except for the para nitro compound, were determined by weighing; the sparingly soluble para nitro compound was determined by absorbance measurements at 265 nm, where the extinction coefficient is 1.15×10^4 O.D. M^{-1} cm^{-1}.[6] All amide solutions were filtered through a millipore filter before use.

K_s for amide binding was determined from the equation[7]

$$K_s = K_D(ED)(amide)/[E_t(D) - K_D(ED) - (ED)(D)]$$

where (ED), (D), and E_t are, respectively, dye–enzyme complex, free dye, and total enzyme concentration, and (amide) is the total amide concentration. This equation is derived for conditions where (amide) > E_t, and neglects contributions from amide–dye interactions. The concentration of enzyme–dye was determined from a value of ΔE_m equal to 1.74×10^4 O.D. M^{-1} cm^{-1} (the average of values given in Table I) and values of K_D at each pH were obtained from the curve in Figure 1.

Since the calculation critically depends upon the determination of the enzyme–dye concentration it is imperative to correct for absorbance associated with all other species. Additional absorbance is contributed by the amide and enzyme, and from interaction of the amide with the enzyme and dye.[3] The principal contribution is from the enzyme, which has an extinction coefficient at 465 nm of 11 O.D. M^{-1} cm^{-1} (determined from 34 separate measurements). Amide absorbance at 465 nm was determined from measurements of amide *vs.* buffer and the extinction coefficients are 0.02, 0.25, 0.57, and 0.07 O.D. M^{-1} cm^{-1}, respectively, for benzamide, *p*-methoxybenzamide, *p*-nitrobenzamide, and furoylamide. Amide–dye plus amide absorbance was determined with each amide (6–15 individual runs) with amide and dye concentrations approximating those used in K_s determinations; absorbance was measured against a dye blank at an identical concentration. Amide–enzyme absorbance was determined from readings of amide–enzyme mixtures (6–15 individual runs) *vs.* a buffer blank; the individual absorbances of amide and enzyme need be subtracted to obtain the amide–enzyme contribution. Poor precision prevented a quantitative treatment of the correction factors in terms of the absorbance of each species and the following empirical relationships were used for correcting the observed optical densities: furoylamide, correction = 1.78(amide) + 0.004; benzamide, correction = 55·(enz) + 0.014; *p*-nitrobenzamide, correction = 55(enz) + 2.85·(amide); *p*-methoxybenzamide, correction = 55(enz) + 0.020. These relationships were derived from studies of amide–enzyme and amide–dye interaction. Although each correction should contain both an enzyme and amide term the limited precision and the relatively small range of amide and enzyme concentrations used precluded the detection of a separate term for each component in all cases. Uncertainty in the correction factors is estimated to be less than 0.005 O.D. The impact of this depends upon what part of the titration curve is being studied; it is minimal near the midpoint. For the results reported here (Table III) K_s (calcd for corrected O.D. + 0.005) − K_s (calcd for corrected O.D. − 0.005)/K_s (calcd for the corrected O.D.) = 0.1–0.3.

Results

Properties of Proflavin. The extinction coefficient of proflavin (3,6-diaminoacridine) monocation (I) was determined from a Beer's law plot at five concentrations of I in the range $0.2–7.9 \times 10^{-6}$ *M*. In ionic strength 0.1 buffers, pH 5.4 and 7.7, and 25°, maximum absorbance was observed at 444 nm and the extinction coefficient is $3.37 \pm 0.18 \times 10^4$ O.D. M^{-1} cm^{-1} (uncertainties are expressed as standard deviations), in excellent agreement with most of the previous determinations.[3,8] Deviations from Beer's law were observed at concentrations of I greater than 10^{-4} *M*; at pH 5.4 and 7.7 the extinction coefficient is equal to $3.18 \pm 0.12 \times 10^4$ for 2×10^{-4} *M* dye. Similar deviations have been observed by other workers.[3,8a,d]

The pK for the first protonation of proflavin to give I has been reported by Albert to be 9.65 at 20° and ionic strength 0.01.[8a] We have redetermined this pK at 25° and ionic strength 0.1 by spectrophotometric titration (at 444 nm) of the dye over the pH range 5–13. Using the method of Albert and Serjeant[9] the calculated pK is 9.49 ± 0.03. Applying the correction for pK change with temperature, given by Albert, to the 20° result predicts a pK of 9.50 at 25°. The extinction coefficient for neutral proflavin at the analytical wavelength (444 nm) is $2.51 \pm 0.03 \times 10^3$ O.D. M^{-1} cm^{-1}; λ_{max} for this species is at 394 nm, where the extinction coefficient is 1.82×10^4 O.D. M^{-1} cm^{-1}.

The pK for diprotonation of proflavin was determined to evaluate the effect of this equilibrium on dye binding to the enzyme at low pH. From absorbance measurements (at 444 nm) with dye concentrations from 0.8 to 1.6×10^{-5} *M*, in the pH range 0.38–1.45 (ionic strength 0.5 with KCl), we calculate a pK of 0.55 ± 0.02. The extinction coefficient for the dication, determined in 4.0 *N* HCl, is 1.08×10^4 O.D. M^{-1} cm^{-1} (at 444 nm). An earlier semiquantitative measurement of the dication equilibrium gives a pK of 1.5 for this reaction.[10] The proflavin dication apparently dimerizes, although to a lesser extent than the monocation I. In 1.92 *M* HCl, where approximately 92% of the dye is dicationic, Beer's law is followed over the range $0.04–1.6 \times 10^{-4}$ *M*; at 4×10^{-4} *M* dye a 6% *positive* deviation from Beer's law was noted.

Enzyme–Dye Binding. Results obtained in a typical experiment are given in Figure 1 and the results are summarized in Table I and Figure 2. The measured absorbances have been corrected for that contributed by uncomplexed enzyme. While this was assumed to be negligible in several earlier studies,[3,8c,d] we have found this not to be the case; the extinction coefficient for the enzyme at 465 nm is reported above.

(5) *Beilstein*, **18**, 276 (1938).
(6) J. T. Edwards and S. C. R. Meacock, *J. Chem. Soc.*, 2000 (1957).
(7) H. Weiner and D. E. Koshland Jr., *J. Biol. Chem.*, 240, 2764 (1965).
(8) (a) A. Albert, "The Acridines," 2nd ed, St. Martin's Press, New York, N. Y., 1966, p 155; (b) F. Millich and G. Oster, *J. Amer. Chem. Soc.*, **81**, 1357 (1959); (c) S. A. Bernhard, B. F. Lee, and Z. H. Tashjian, *J. Mol. Biol.*, **18**, 405 (1966); (d) A. N. Glazer, *Proc. Nat. Acad. Sci. U. S.*, 54, 171 (1965); (e) G. R. Haugen and W. H. Melhuish, *Trans. Faraday Soc.*, 60, 386 (1964); (f) D. P. Craig and L. N. Short, *J. Chem. Soc.*, 419 (1945).
(9) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Wiley, New York, N. Y., 1962, p 73.
(10) G. R. Haugen and W. H. Melhuish, *Trans. Faraday Soc.*, 60, 386 (1964).

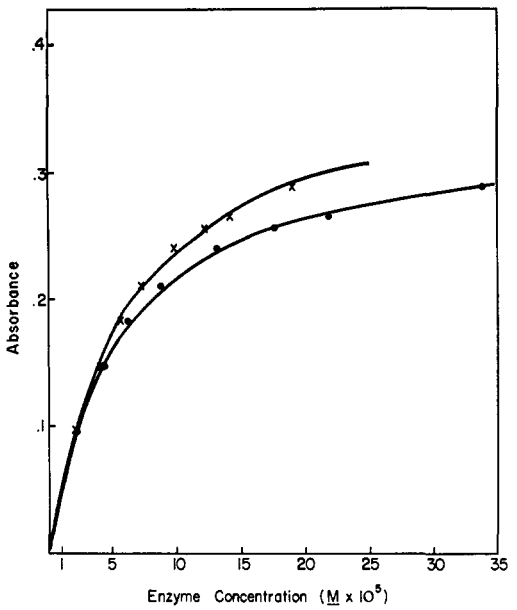

Figure 1. Dye binding at pH 6.10. The curves are computre calculated for binding to the enzyme monomer[17] (×), and for bin-d ing to the enzyme without any correction for enzyme polymerization (●).

The K_D values reported here are in reasonable accord with most of those obtained previously (Table II) under similar conditions. A significant element accounting for the differences is in the correction for enzyme absorption. The difference extinction coefficient is independent of pH (Table I) and the average value, equal to 1.74×10^4, is in good agreement with the 1.85×10^4 reported previously,[8c] but differs substantially from the 2.6×10^4 reported by Brant, *et al.*[3]

Table I. Dissociation Constants and Difference Extinction Coefficients (ΔE_m) of the Proflavin–Chymotrypsin Complex at 25° and Ionic Strength 0.1[a]

pH	Buffer	Enzyme range $M \times 10^4$	K_D[b] $M \times 10^5$	ΔE_{max}[b] O.D. $M^{-1}cm^{-1} \times 10^{-4}$
3.25	Glycine	1.4–2.4	47.8	1.75
3.36	Formate	0.5–3.2	15.36 (1.0)	1.72 (0.05)
3.89	Formate	0.9–3.1	11.57 (1.0)	1.80 (0.06)
3.91	Formate	0.5–3.7	12.81 (0.87)	1.85 (0.05)
4.34	Formate	0.5–3.0	9.28 (1.0)	1.79 (0.06)
5.00	Acetate	0.4–3.3	9.89 (0.52)	1.65 (0.03)
5.02	Acetate	0.5–3.5	10.76 (0.78)	1.63 (0.04)
5.43	Acetate	0.2–4.5	8.18 (0.66)	1.82 (0.05)
5.43	Acetate	0.2–4.5	9.19 (1.1)	1.88 (0.08)
5.79	Phosphate	0.2–2.1	5.13 (0.42)	1.58 (0.04)
5.79	Phosphate	0.4–3.4	5.42 (0.20)	1.60 (0.01)
5.88	Phosphate	1.0–3.0	6.00 (0.20)	1.68 (0.09)
6.01	Phosphate	0.3–3.4	5.74 (0.32)	1.65 (0.03)
6.10	Phosphate	0.2–3.4	5.41 (0.20)	1.67 (0.02)
6.42	Phosphate	0.2–3.3	4.90 (0.32)	1.79 (0.03)
6.94	Phosphate	0.2–3.3	3.47 (0.40)	1.82 (0.02)
7.31	Phosphate	0.2–3.5	3.16 (0.20)	1.88 (0.03)
7.73	Tris	0.3–3.6	3.35 (0.18)	1.84 (0.02)

[a] Reactions were run with six–eight enzyme concentrations over the range stated, except for the reaction at the lowest pH where only three points were recorded. pH variations were almost always less than 0.02 unit, and the buffer concentration was adjusted so that the ionic strength contributed by charged buffer species was 0.1. The dye concentration was 4×10^{-6} *M*. [b] The K_D and ΔE_{max} values are followed by the computer-calculated standard error.

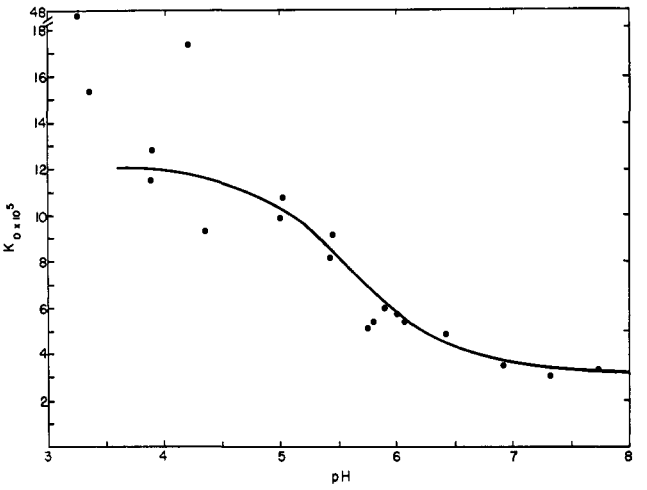

Figure 2. pH dependence of proflavin binding to chymotrypsin at 25°. The line is drawn for dissociation constants equal to 12.19×10^{-5} and 3.25×10^{-5} for an acid–base pair with a pK of 5.60.

The trend for higher values for K_D at lower pH observed by others[3,8d] has been resolved into an interpretable pH profile in which a group on the enzyme with an apparent pK of 5.60 affects proflavin binding; K_D for the conjugate base is 3.25×10^{-5} and K_D for the conjugate acid is 12.19×10^{-5}. At pH's below 4 the K_D increases markedly until at pH 3.35 this constant approximately doubles with a 0.1-unit decrease in pH. An experiment, summarized in Figure 3, shows that temperature control is imperative at low pH; the absorbance at more alkaline pH's was insensitive to these small temperature changes. Temperature was measured with a calibrated thermocouple immersed directly in the sample cuvette under study. No identification was made on the source of the temperature-dependent spectral effects which predominate at low pH. The stability of the absorbance in the neutral pH range is noteworthy since it supports the notion that significant autolysis does not occur during the time necessary for the absorbance measurements. Shiao and Sturtevant's calorimetric studies of chymotrypsin solutions

Table II. Published Values of K_D for the Proflavin–Chymotrypsin Complex

pH	$K_D \times 10^5$	Conditions	Ref
8.0	2.7 ± 0.1	Ionic strength 0.39, 24°	*a*
6.0	4.4 ± 0.4		
5.7	4.3 ± 0.4		
5.4	3.3 ± 0.2		
5.0	3.9 ± 0.2		
4.6	7.3 ± 0.5		
4.3	7.9 ± 0.4		
4.0	8.4 ± 0.6		
2.5	14.1 ± 0.8		
7.6	2.2 ± 0.5	0.2 *M* phosphate	*b*
8.0	3.2, 3.7 ± 0.3	Ionic strength 0.1, 25°	*c*
7.0	2.5	0.05 *M* phosphate, 2°	*d*
7.9	13	Ionic strength 0.1, 25°	*e*

[a] Reference 3. [b] Reference 8d. [c] Reference 8c. [d] Reference 7. [e] R. A. Wallace, A. N. Kurtz, and C. Niemann, *Biochemistry*, **2**, 824 (1963).

Table III. Amide Binding to Chymotrypsin at 25° [a]

Amide	pH	Amide × 10^2, M	Enzyme × 10^4, M	No. experiments	$K_s \times 10^3$, M [b]	Calcd range [c] × 10^3, M
Benzamide	5.79–5.86	1.3–6.0	1.4–3.4	10	2.83 (0.55)	2.51–3.15
	7.69–7.73	4.7–7.0	1.1–3.4	15	3.06 (0.43)	2.86–3.26
p-Nitrobenzamide	5.78–5.90	3.1	1.0	10	0.749 (0.19)	0.639–0.859
	7.64–7.79	2.7–3.9	0.9–2.3	11	1.34 (0.35)	1.15–1.53
p-Methoxybenzamide	5.75–5.96	0.9–1.3	0.9	11	1.22 (0.28)	
	7.71–7.80	1.2	0.8–2.1	11	1.38 (0.43)	
Furoylamide	5.83–5.93	0.6–4.5	1.5–2.4	14	14.01 (2.3)	12.91–15.11
	7.70–7.76	2.3–3.0	1.5	9	13.86 (4.0)	11.33–16.39

[a] Reaction conditions were equivalent to those described for Table I. [b] The standard error follows K_s. [c] Calculated for p of 0.9.

at pH 7.8, 25°, and ionic strength 0.25 show that enzyme solutions of the concentrations used here are stable for at least 45 min.[11]

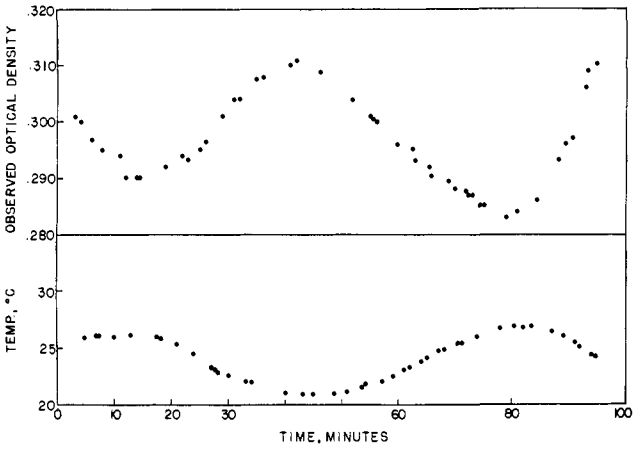

Figure 3. Difference optical density as a function of time and temperature for 4×10^{-6} M proflavin and 3×10^{-4} M enzyme at pH 3.3. The lower curve plots the temperature of the solution under study and the upper curve plots the optical density.

Amide Binding. Dissociation constants for the reaction of amides with chymotrypsin are given in Table III. The principle finding is that except for the para nitro compound binding occurs to the same extent at pH's 7.7 and 5.8. The nitro compound is bound 1.8-fold more tightly at the lower pH; a Student's t test gives p equal to 0.98 for this difference, and the calculated range for the ratio of equilibrium constants at pH's 7.7 and 5.8 is 1.40–2.35 for p equal to 0.9.[12] Results obtained with *p*-methoxybenzamide were rather erratic in that while the standard error for experiments done on a single day was extremely small, some unidentified systematic error resulted in wide variations in runs done on separate days. The values given for this compound in Table III are, therefore, of a semiquantitative nature. We estimate the uncertainty here at less than 50%.

Discussion

Effects of Enzyme Polymerization on Dye Binding. Inherent in an approach involving saturation of substrate (proflavin) with enzyme is the potential for effects of enzyme polymerization on substrate affinity. The reverse procedure, substrate saturation of the enzyme, is precluded in studies with proflavin, at least under conditions where the affinity for dye is poor, since the dye polymerizes at very low concentrations (see above). It is known that chymotrypsin polymerizes,[13] but there is conflicting evidence as to how this affects the substrate binding site. Supporting the view that dimerization and trimerization are without effect on binding are the observations: (a) that the ultracentrifugation patterns of the enzyme are unaffected by an enzyme inhibitor, β-phenylpropionate,[14] (b) that the enzyme dimer present at pH 6.2 is not without catalytic activity,[15] (c) that an equivalent binding constant for proflavin is obtained at pH 8 when a low concentration of enzyme is saturated with dye and when the dye is saturated with enzyme.[3] In the latter studies the enzyme concentration went into the range where polymerization is expected to occur. A conflicting conclusion may be derived from results obtained in studies of the enzyme concentration dependence of the enthalpy of substrate binding,[13i] and the kinetics of the chymotrypsin–proflavin reaction.[16] The latter study provides especially convincing evidence that polymeric states of the enzyme have a decreased capacity for proflavin binding.

Since the equilibrium constants for chymotrypsin dimerization and trimerization are not available for the conditions used in experiments reported here, we were unable to correct for these reactions. Using the constants for pH 6.2, phosphate buffer, ionic strength 0.2,[13g] the enzyme monomer concentration was calculated[17] and the results obtained in dye-binding studies at pH's near 6.2 were analyzed using this concentration. The results so obtained for a reaction at pH 6.10 are given in Figure 1; the calculated K_D and ΔE_M are 1.16 and 1.15 times, respectively, the values obtained without such correction. It was found that for all cases, the computer-calculated standard error for both parameters is larger when the enzyme monomer

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(17) We are indebted to Professor L. D. Faller for providing these values.

concentration was used for calculation. This may come from the fact that in the monomer-corrected runs the enzyme concentration covered a smaller fraction of the titration curve; statistical certainty increases when the per cent saturation increases. Also, polymeric forms of the enzyme might possibly bind dye, or perhaps the equilibrium constants used for calculation of the monomer concentration might be inappropriate for the conditions used here.

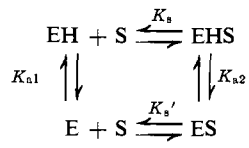
Comparison of the monomer corrected and uncorrected values for K_D strongly suggest that relatively little uncertainty is generated by our inability to definitively deal with the question of enzyme polymerization. The error introduced by this factor appears to be no greater than due to the small experimental error; standard errors were ordinarily less than 10% (Table I).

pH Effects of Dye Binding. The pH dependence for dye binding (Figure 2) in the near-neutral pH range may be accounted for by assuming that protonation of the active-site histidine residue decreases the binding of the cationic dye.

For Scheme I

$$K_{obsd} = \frac{K_s'K_{a2} + K_s(H)}{K_{a2} + (H)} \quad (1)$$

Scheme I



with all constants written in the direction for dissociation. Equation 1 describes a sigmoid curve, either ascending or descending, depending on the relative size of K_s and K_s'. At high pH, $K_{obsd} = K_s'$; at low pH, $K_{obsd} = K_s$ and the midpoint of the pH profile occurs at a pH equal to pK_2. From the relationship

$$K_s/K_s' = K_{a2}/K_{a1} \quad (2)$$

the dissociation constant of the free enzyme (K_{a1}) may be calculated from the three constants obtained from the pH profile (Figure 2); K_{a1} is equal to 6.68×10^{-7} (pK 6.18). This is significantly lower than most of the kinetically determined values for K_{a1}, which fall in the range 6.3–7.2.[18]

The decreased binding below pH 3.5 may reflect the effect of pH on the Asp-194–Ileu-16 hydrogen bond, which is responsible for holding the enzyme in a catalytically active conformation.[19,20] The high-temperature sensitivity in this pH range suggests that proton association is coupled with some other reaction having a high enthalpy change since the enthalpy for proton reaction with carboxylic acids is ordinarily about zero.[21] Since diprotonation of proflavin occurs at a very low pH (pK 0.55), it may be concluded that this reaction does not account for the decreased binding at pH 3.5 and below.

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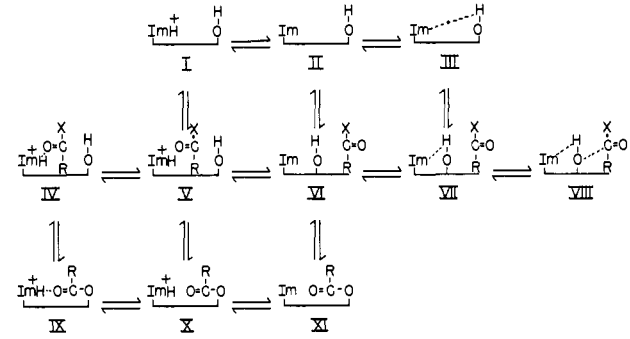
The pK of Histidine-57 in Chymotrypsin. Scheme II[22] shows possible interactions which may influence the

Scheme II



ionization of histidine in the free enzyme (top row), the Michaelis complex (middle row), and acyl enzyme (bottom row). The importance of structure III, in which the histidine conjugate base is hydrogen bonded to Ser-195, is suggested by the low pK of the free enzyme (pK 6.18, see above); an orientation appropriate for this bonding has been seen in X-ray structure studies.[20] The hydrogen bond in IX (with an intervening water molecule) has been observed in an X-ray study of indoleacryloylchymotrypsin.[22f]

The free-energy change for the reactions II–III and X–IX can be calculated if the pK of the uncomplexed histidine residue (I and X) is known. The kinetically determined pK for 3,5-dinitrobenzoylchymotrypsin is 6.95, and this is lower than that observed for benzoyl-, *p*-nitrobenzoyl-, *p*-methoxybenzoyl-, and furoylchymotrypsin;[2b] there is a reasonably linear relationship between the apparent pK for activity and electron withdrawal by the acyl group[22h] (as measured by the pK of the carboxylic acid[2b,c]). The equilibrium X–IX will be disfavored by electron withdrawal and if it is assumed that hydrogen bonding is negligible with the 3,5-dinitrobenzoyl enzyme, then the pK of X is equal to 6.95. It is lower if hydrogen bonding with the dinitro derivative is still significant. If the pK

(22) (a) Scheme II has been simplified by omission of a structure in which the His-57 conjugate acid acts as a proton donor in a hydrogen bond to Ser-195. This omission is based upon the following. The pK of the enzyme is low compared to that of free histidine suggesting that the reaction in which His-57 acts as a proton acceptor (II–III) is *relatively* more important than a reaction in which the conjugate acid acts as a proton donor. This is expected since the strength of a hydrogen bond is inversely proportional to the difference in pK of the proton acceptor and donor.[22b] This difference is approximately 7 pK units for the II–III reaction and about 10 pK units for the reaction involving the His-57 conjugate acid and the serine hydroxyl.[22c] Certainly if hydrogen bonding in IX is significant, then the corresponding hydrogen bond to the more basic serine hydroxyl will be stable. What is important, however, is that this reaction is probably not significant compared with the II–III process. The hydrogen bonding of His-57 with Asp-102[22e] has also been omitted since this bonding is not directed to the site of acylation or to the substrate and it might be expected that this process will not be significantly influenced by enzyme acylation or substrate binding. The Asp–His hydrogen bonding probably increases the basicity of the histidine group since the stronger hydrogen bond is expected for the histidine conjugate acid. Again, the low pK of the enzyme supports the view that the II–III reaction is a relatively more favorable process. (b) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p 326. (c) The pK's of aliphatic alcohols are in the range -2 to -4.[22d] (d) E. M. Arnett, *Progr. Phys. Org. Chem.*, **1**, 223 (1963). (e) D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature (London)*, **221**, 337 (1969). (f) R. Henderson, *J. Mol. Biol.*, **54**, 341 (1970). (g) This shift will not depend upon the reactions omitted from Scheme II;[22a] if all interactions in the free enzyme are preserved in the Michaelis complex, formation of IV will simply add to the stabilization of the His-57 conjugate acid. (h) This is good evidence against the possibility that the pK variations are caused by the hydrophobic environment provided by the acyl group.

of I is identical with that of X, the free-energy change for reaction II–III is −1.05 kcal/mol (25°) (based on pK 6.95). This change for reaction X–IX depends on the acyl group and based upon a pK of 6.95 for X, it is −0.20, −0.55, −0.36, and −0.50 kcal/mol (25°) for furoyl- (pK 7.10), benzoyl- (pK 7.36), *p*-nitrobenzoyl- (pK 7.22), and *p*-methoxybenzoyl- (pK 7.32) chymotrypsin, respectively. The calculated free-energy change for the II–III reaction may be less negative if the pK of I is lower than 6.95, and the values for the acyl enzyme reaction X–IX may be more negative, for the same reason.

The pK of the histidine residue in the Michaelis complex V may be shifted—relative to the free enzyme I—upward by the reaction V–IV, or downward by the reaction VII–VIII. We have previously found a substituent-dependent downward pK shift with acetyltryptophan anilides[2a] and these results were interpreted in terms of a mechanism involving an equilibrium formation of a tetrahedral addition compound to which the histidine conjugate base forms a strong hydrogen bond. The structure proposed was similar to that given in VIII, except that the bond between the serine oxygen and carbonyl group was presumed to be covalent. The pK perturbing effect of the VII–VIII reaction will be independent of the nature of bond here; what is important is that the reaction be an equilibrium process; VIII must go to re-form VII faster than it breaks down to some other product.

In binding of substituted benzamides and furoylamide the His-57 pK may be shifted upward by reaction V–IV,[22g] or downward by reaction VII–VIII. The latter is not expected since these compounds do not acylate the enzyme and it is our assumption that VIII is an intermediate on the path for enzyme acylation.[23a] If the V–IV reactions in noncovalent complexes are equivalent to the X–IX reactions in the corresponding acyl enzymes, then the His-57 pK will be perturbed by binding these inhibitors; this will reflect itself by substrate binding being pH dependent. Assuming a pK of 6.95 for I and V, that the II–III and VI–VII reactions are identical, that VII–VIII is negligible, and that the V–IV equilibrium is described by the X–IX equilibrium constants calculated for the corresponding acyl enzymes, then the observed dissociation constant for furoylamide, benzamide, and *p*-nitrobenzamide will be 1.30, 1.99, and 1.60 times, respectively, as large at pH 7.7 as at pH 5.8.[23b] This is not observed (Table III). We are reluctant to speculate on the results obtained with the para nitro compound since there is an important additional unknown parameter involved in reactions of this derivative as reflected by the fact that the rate of hydrolysis of *p*-nitrobenzoylchymotrypsin shows an approximately 35-fold negative deviation from the Hammett relationship established with related compounds.[24] The failure of the V–IV reaction to be manifest with furoylamide and benzamide may be taken to indicate that these acyl groups are bound to a different locus than in the corresponding acyl enzymes. Perhaps even slight misorientation prevents the pK perturbing reaction.

Other Studies of the Histidine-57 pK. Results of studies of proton release on substrate binding to chymotrypsin generally indicate that covalent linkage to Ser-195 is required for significant modification of the His-57 pK. For example, the change in pK induced by covalent linkage to the following groups is: 6.6–7.4 with diphenylcarbamylation,[25] 6.4–7.3 with acetylation,[26] 6.3–7.7 with indoleacryloylation;[27] no change occurs after reaction with phenylmethanesulfonyl fluoride.[28] Except for the last study, these results may be taken to indicate that the II–III reaction, which is abolished by acylation, is responsible for the high acidity of His-57. With noncovalent binding in the *neutral* pH range (pH 6–7.5) there are no protons released on reaction with indole and phenol,[29] and 0.14 proton taken up on binding acetyl-D-tryptophan.[29] Scheme II does not predict any proton release in this pH range if the IV–V and VII–VIII reactions are negligible. Anisole binding shifts a pK from 6.7 to 6.4 and formanilide displaces an unidentified pK from 5.5 to 5.0.[30] Also, inhibitor binding to chymotrypsin in the neutral pH range with compounds containing an aromatic and/or carbonyl group is moderately pH dependent;[31] perhaps equilibrium VII–VIII contributes here since the binding becomes weaker at lower pH; this is the direction predicted for involvement of the VII–VIII reaction. It should be noted that results obtained in studies of proton release must be interpreted with caution since such studies have generally been done with very high enzyme concentrations and no account has been taken of the effects of substrate binding on the pH-dependent enzyme polymerization.[32,33] Unless the polymerization equilibria are unaffected by substrate binding, which may not be true for noncovalent binding (see above), and has only been shown to be the case for the acetylated enzyme at pH 4,[13h] results of such studies may be influenced by substrate effects on the polymeric state of the enzyme.

(23) (a) Strong support for our proposed new intermediate **2a** has recently come from the direct observation of an equilibrium process located on the reaction scheme between the acyl enzyme and Michaelis complex in the reaction of chymotrypsin with acetyltryptophanamide (**20a**). (b) These are calculated from eq 1 and 2 and Scheme I where S is equal to amide.

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(33) It has previously been pointed out that proton release associated with acetylation may be complicated by the fact that the state of the enzyme may be different at different pH's.[34]
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