Binding of Proflavine to α-Chymotrypsin and Trypsin and Its Displacement by Avian Ovomucoids*

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ABSTRACT: Proflavine is an inhibitor of α-chymotrypsin and trypsin. When it is bound to either enzyme there is a shift of its absorption spectrum to a longer wavelength. Avian ovomucoids inhibit proteases such as α-chymotrypsin or trypsin. When those ovomucoids which are inhibitors of α-chymotrypsin were added to the proflavine–chymotrypsin complex, the spectral shift was eliminated stoichiometrically. Chicken ovomucoid, which does not inhibit chymotrypsin, did not affect the spectral shift. Similar results were obtained with ovomucoids and the proflavine–trypsin complex. Turkey ovomucoid inhibits both α-chymotrypsin and trypsin. The rates of the interactions of turkey ovomucoid with α-chymotrypsin or trypsin were studied spectrophotometrically in a stopped-flow apparatus, using displacement of proflavine. It was found that turkey ovomucoid reacted faster with chymotrypsin than with trypsin. This is in agreement with previously reported relative rates obtained by measuring catalytic activities. The effect of pH on the binding of proflavine to α-chymotrypsin was studied spectrophotometrically. Below pH 4, the binding strength decreased markedly. It is suggested that an ionization of a carboxylic acid side chain near the binding site of α-chymotrypsin affects the binding of proflavine.

Wallace *et al.* (1963) found that proflavine is a competitive inhibitor of α-chymotrypsin. Bernhard and Lee (1964) reported that, as proflavine becomes bound to α-chymotrypsin, there is a shift of its absorption spectrum toward longer wavelengths. This spectral shift was used by Bernhard and Lee (1964) to calculate the dissociation constant (K_{diss}) of the proflavine–chymotrypsin complex. They found that K_{diss} is equal to K_I for the inhibition of the hydrolysis of a substrate by α-chymotrypsin. This indicates that proflavine is bound to the substrate binding site of α-chymotrypsin. Weiner and Koshland (1965), Glazer (1965), and Bernhard *et al.* (1966) have shown that the binding is stoichiometric, namely that 1 mole of proflavine is bound to 1 mole of α-chymotrypsin. Glazer (1965) and Bernhard *et al.* (1966) reported a spectral shift to longer wavelengths when proflavine is mixed with trypsin. They also found that the binding is stoichiometric. Bernhard and Gutfreund (1965) used proflavine as a probe to investigate the kinetics of reactions catalyzed by trypsin, while Brandt and Hess (1966) used proflavine to determine the binding constant of a specific substrate to α-chymotrypsin.

Avian ovomucoids have the capacity to inhibit proteolytic enzymes such as α-chymotrypsin or trypsin or both (Rhodes *et al.*, 1960; Stevens and Feeney, 1963). For example, Rhodes *et al.* (1960) found that chicken ovomucoid inhibits trypsin, while golden pheasant ovomucoid inhibits α-chymotrypsin, and turkey ovomucoid[1] inhibits both trypsin and α-chymotrypsin. Several different methods have been used to study the interactions of ovomucoids and other proteinaceous inhibitors with proteolytic enzymes (Laskowski and Laskowski, 1954). Green (1953) and Simlot and Feeney (1966) used enzymatic assays to study such interactions. Rhodes *et al.* (1960) and Balls and Ryan (1963) used ultracentrifugal techniques. Lebowitz and Laskowski (1962) used potentiometric titration, while Edelhoch and Steiner (1965) used a fluorescence quenching technique, and Osuga and Feeney (1967) employed gel electrophoresis.

In the present work we have used the spectral shifts of proflavine to study the interaction of several ovomucoids with α-chymotrypsin and trypsin. In determining the permissible parameters, the effect of pH on the binding of proflavine to α-chymotrypsin was studied.

Experimental Section

Materials. The bovine α-chymotrypsin (salt free, three times crystallized) and trypsin (salt free, two times crystallized) were purchased from Worthington Biochemical Co. Proflavine dihydrochloride (mol wt 318.2)

* From the Department of Food Science and Technology, University of California, Davis, California. *Received October 5, 1966.* This work was supported in part by Research Grants AI-03484 and HD-00122 from the National Institutes of Health, U. S. Public Health Service. Presented in part at the 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 12–16, 1966.

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[1] Turkey ovomucoid is capable of inhibiting α-chymotrypsin or trypsin or both simultaneously (Rhodes *et al.*, 1960; Stevens and Feeney, 1963; Simlot and Feeney, 1966).

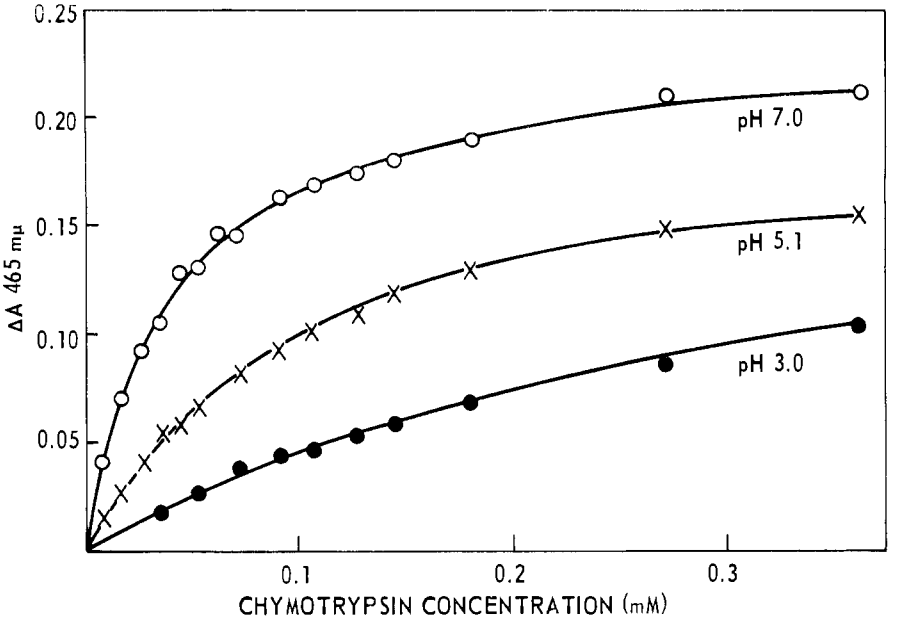

FIGURE 1: Titration of proflavine with α-chymotrypsin. The concentration of proflavine was 0.01 mM. Other details of the titration experiments are given in the text.

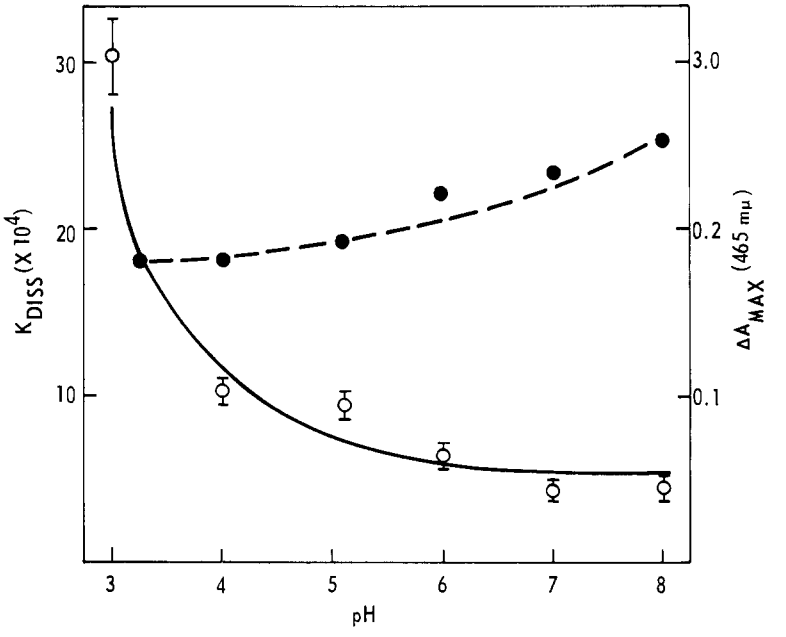

FIGURE 2: The effect of pH on the binding of proflavine to α-chymotrypsin. The concentration of proflavine was 0.01 mM. Other conditions are described in the text. (●) ΔA_{max} extrapolated to infinite concentrations of α-chymotrypsin; (○) dissociation constant (K_{diss}) for proflavine–chymotrypsin.

was purchased from Mann Research Laboratories, Inc. Ovomucoids were isolated from the egg whites by the trichloroacetic acid–acetone method of Lineweaver and Murray (1947) and purified by chromatography on CM-cellulose and DEAE-cellulose (Rhodes *et al.*, 1960; Stevens and Feeney, 1963). The egg whites were from the chicken (*Gallus gallus*), turkey (*Meleagres gallopavo*), and golden pheasant (*Chrysolophus pictus*).

Proflavine Titrations. Stock solutions of proflavine were prepared at weekly intervals and stored in the dark. The absorption maximum and extinction coefficients were obtained with 0.01 mM solutions of proflavine in 0.1 M sodium phosphate buffer, pH 7.0.

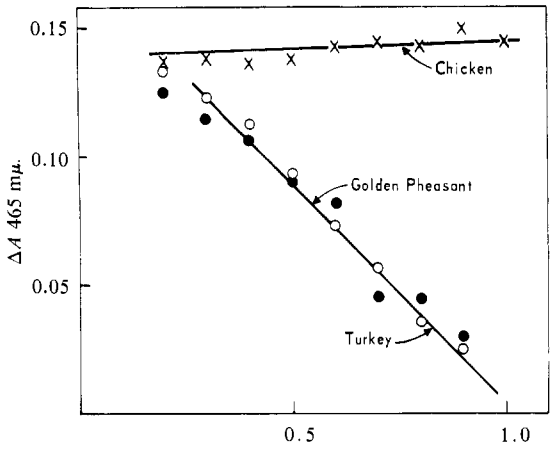

FIGURE 3: Displacement of proflavine from α-chymotrypsin by avian ovomucoids at pH 7.0. The concentrations of proflavine and α-chymotrypsin were 0.01 and 0.1 mM, respectively. Other conditions are described in the text. No attempt was made to plot the values for lower ratios of ovomucoid to α-chymotrypsin. At very low ratios of turkey or golden pheasant ovomucoids to α-chymotrypsin, the conditions of equilibrium between proflavine and α-chymotrypsin are such that linearity may not exist. This effect is evident from the plots of Figure 1. (○) Golden pheasant ovomucoid; (●) turkey ovomucoid; (×) chicken ovomucoid.

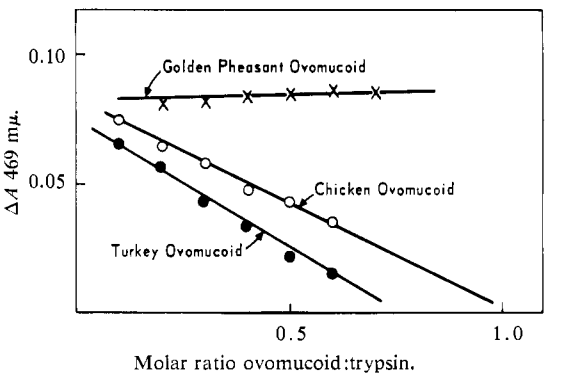

FIGURE 4: Displacement of proflavine from trypsin at pH 7.0. The concentrations of proflavine and trypsin were 0.01 and 0.2 mM, respectively. Other conditions are described in the text. (×) Golden pheasant ovomucoid; (●) turkey ovomucoid; (○) chicken ovomucoid.

The maximum was at 444 mμ and $E_{444m\mu}$ was 41,000. The differences in absorption were measured at 465 (with α-chymotrypsin) and at 469 mμ (with trypsin) (Glazer, 1965). Variation of pH was not found to affect the wavelength at which the maximum difference in extinction was obtained when proflavine was titrated with α-chymotrypsin. Solutions of α-chymotrypsin and trypsin were prepared in the appropriate buffers and used within 30 min after preparation. The concentrations of α-chymotrypsin solutions were determined spectrophotometrically at 282 mμ using the factor of 2.075 (Schwert and Kaufman, 1951), and the concentrations of the trypsin solutions were determined at 280 mμ using the factor of 1.533 (Laskowski and Laskowski, 1954). The following buffers were used: pH 3–5.1, 0.1 M sodium acetate; pH 6 and 7, 0.1 M sodium phosphate; pH 8, 0.1 M Tris-HCl. At a given pH, all the reactants were dissolved in 0.1 M solutions of the appropriate buffers. Absorption difference measurements were made with a Bausch and Lomb recording spectrophotometer, Spectronic 600, with matched 1.0-cm cells.

Rate Measurements. Measurements of the displacement of proflavine from α-chymotrypsin were done spectrophotometrically in a Durrum–Gibson-type, stopped-flow apparatus. The rapid mixing was completed within 10 msec and the per cent transmission was determined at the appropriate wavelength (465 mμ with α-chymotrypsin and 469 mμ with trypsin). The change in the per cent transmission was recorded by photographing the patterns on an oscilloscope. The final concentrations in the reaction mixtures in the rate measurements with α-chymotrypsin were as follows: 0.1 mM, α-chymotrypsin; 0.01 mM, proflavine; and 0.1 mM, turkey ovomucoid. With trypsin the final concentrations were as follows; 0.2 mM, trypsin; 0.012 mM, proflavine; and 0.2 mM, turkey ovomucoid.

Results

Effect of pH on Proflavine Binding to α-Chymotrypsin. When proflavine was titrated with α-chymotrypsin, the greatest spectral difference in the pH range from 3 to 8 was at 465 mμ. The magnitude of the spectral difference at 465 mμ was pH dependent (Figure 1). At any given concentration of α-chymotrypsin, an increase in the pH caused a greater spectral difference. At any given pH value, an increase in the concentration of α-chymotrypsin caused an increase in the spectral difference. The maximum spectral difference (ΔA_{max}) at any given pH was calculated by a Scatchard (1949) plot with the aid of a high-speed IBM computer. The K_{diss} at any given pH was calculated from the equation, K_{diss} = ([proflavine]/[proflavine–chymotrypsin])[chymotrypsin] = $([\Delta A_{max} - \Delta A]/[\Delta A])[E_0 - C_0(\Delta A/\Delta A_{max})]$, in which ΔA is the observed spectral difference; E_0 the initial concentration of α-chymotrypsin; and C_0 the initial concentration of proflavine. The values for calculated ΔA_{max} and K_{diss} as functions of pH are shown in Figure 2. There was a slight increase in the ΔA_{max} values when the pH was increased from 3 to 8, but the increase in the ΔA_{max} was only very little between pH 3 and 4. However, the changes (decreases) in K_{diss} were much greater. The decrease in K_{diss} when the pH was increased from 3 to 4 was significantly greater than the change in ΔA_{max}.

Displacement of Proflavine from α-Chymotrypsin

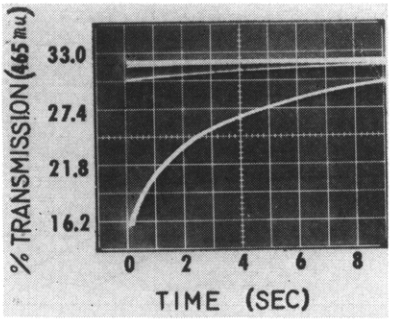

FIGURE 5: Stopped-flow determinations of the reaction between turkey ovomucoid and proflavine–chymotrypsin mixture. Final concentrations in reaction mixture were 0.01, 0.1, and 0.1 mM for proflavine, α-chymotrypsin, and turkey ovomucoid, respectively. Other conditions are described in text.

and Trypsin by Ovomucoids. Different ovomucoids were added to a mixture of proflavine and α-chymotrypsin and the interaction of the ovomucoids with the enzyme was followed spectrophotometrically (Figure 3). When graduated amounts of turkey or golden pheasant ovomucoids, which are inhibitors of α-chymotrypsin, were added to the proflavine–chymotrypsin mixture, there were corresponding quantitative decreases in the spectral difference. However, chicken ovomucoid, which does not inhibit α-chymotrypsin, did not affect the spectral difference. The results of similar studies with trypsin are shown in Figure 4. When chicken and turkey ovomucoids, which are inhibitors of trypsin, were added to the proflavine–trypsin mixture, there was a quantitative decrease in the spectral difference. When golden pheasant ovomucoid, which does not inhibit trypsin, was added, no detectable spectral difference was found.

Rate Measurements. The rate of the elimination of the spectral difference in proflavine–chymotrypsin mixtures upon addition of turkey ovomucoid was determined spectrophotometrically in a stopped-flow apparatus. The oscilloscope tracing of a stopped-flow experiment done at pH 7.0 is shown in Figure 5. The time required for the elimination of one-half of the spectral difference, $t_{1/2}$, was 1.7 sec when turkey ovomucoid was added to an equivalent amount of α-chymotrypsin.[2] The second-order rate constant, k_2, was calculated, using K_{diss} of 4.18×10^{-5} M for proflavine–chymotrypsin. The value of k_2 was 1.62×10^4 mole $l.^{-1}$ sec^{-1}. An attempt was made to determine k_2 for the interaction of turkey ovomucoid with trypsin. However, owing to a continuously increasing base line, apparently caused by continuous autolysis of trypsin in 0.1 M phosphate at pH 7.0, we could only determine that $t_{1/2}$ was 0.2 ± 0.04 sec.

Discussion

Bernhard and Lee (1964) reported a solvent effect on the absorption spectrum of proflavine. They noted a shift of the absorption maxima when proflavine was transferred from aqueous solution to more apolar solvent. Since a similar shift occurred when α-chymotrypsin was added to proflavine solution, Bernhard and Lee (1964) and Glazer (1965) concluded that the binding site of α-chymotrypsin has an apolar character. This conclusion is also supported by the work of Kallos and Avatis (1966). Deranleau and Neurath (1966) reported only a slight change (<15%) in the K_{diss} of a synthetic inhibitor of α-chymotrypsin between pH 3.3 and 5.5. In the present study we found that the affinity of proflavine for α-chymotrypsin was markedly decreased when the pH was lowered below 4.0. On the other hand, there was only a slight change in the ΔA_{max} with pH changes. The fact that ΔA_{max} does not change much with pH change indicates that there is only slight change in its environment, namely in the binding site of α-chymotrypsin. Sarfare *et al.* (1966) recently have demonstrated that polymerization of α-chymotrypsin does not affect the binding of small molecules by the enzymes. So it might be concluded that the decrease in the K_{diss} observed in this study resulted from change in K_{diss} and not from polymerization of α-chymotrypsin. Vaslow (1958) suggested that there is a positive charge in the active center of α-chymotrypsin. In contrast, Wallace *et al.* (1963) concluded that there is a negative charge in close proximity to the binding site of α-chymotrypsin, and Stewart *et al.* (1963) suggested that there is a functional carboxyl group in α-chymotrypsin. The results of the present study tend to support the hypothesis that there is a negative charge in the vicinity of the binding site of α-chymotrypsin. The fact that the greatest change in the affinity of proflavine occurs between pH 3 and 4 indicates that there may be carboxylic acid side chains in the vicinity of the active site. These latter conclusions are based on the assumptions that the proflavine is bound, at least in part, on the same sites that substrate is bound. However, the proflavine might be bound elsewhere on the molecule and the effects of its binding then would necessarily be secondary, such as causing a conformational change unsuitable for substrate binding. If this were the case, then the data for K_{diss} would only concern the proflavine binding site. A still further possibility is that a conformational change might occur in this pH range and then dissociation of the proflavine might be caused by the conformational change.

We found that proflavine is a useful tool for studying the interaction of protein inhibitors with α-chymotrypsin and trypsin. It can be used for determining both

[2] According to B. H. Havsteen and M. Eigen (a personal communication quoted by Brandt and Hess, 1966) the dissociation rate constant for the proflavine–chymotrypsin complex is 250 sec^{-1}. Brandt and Hess stated that this dissociation due to mere dilution was too fast to be measured. Probably also in our rate experiments in the stopped-flow apparatus the spectral change due to dilution occurred within a very short period of time, outside of our resolution time. It therefore appears most probable that the rate of proflavine displacement was imposed by the rate of ovomucoid binding.

stoichiometry and specificity. Rhodes *et al.* (1960) reported that chicken ovomucoid inhibits trypsin but not α-chymotrypsin. Chicken ovomucoid displaced proflavine quantitatively from trypsin, but it did not displace proflavine from α-chymotrypsin. Similar results confirming the specificities (Rhodes *et al.*, 1960; Stevens and Feeney, 1963) of golden pheasant and turkey ovomucoids were obtained during this investigation. Simlot and Feeney (1966) using enzymatic assays reported that turkey ovomucoid inhibits trypsin faster than it inhibits α-chymotrypsin. The rate measurements obtained with the stopped-flow apparatus confirmed and quantitated this observation. With the values of $t_{1/2}$ as criteria, the rate of reaction of turkey ovomucoid with trypsin appears to be seven to ten times faster than the rate of reaction of turkey ovomucoid with α-chymotrypsin.

Acknowledgments

We wish to thank Dr. L. L. Ingraham and Dr. J. Whitaker for advice on calculations and mechanism, F. C. Greene for assistance with the calculations done by the computer, and Dr. J. H. Swinehart for making the stopped-flow apparatus available and assisting in its use.

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