plex the relaxation event is also site specific (Lovett *et al.*, 1974a). Furthermore, the site of the nick in the case of the ColE1 plasmid appears to be located at the origin of ColE1 DNA replication (Lovett *et al.*, 1974b). Whether this characteristic is also shared by the R6K plasmid is currently under investigation.

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Proton Binding Changes in α-Chymotrypsin Dimerization†

Thomas A. Horbett and David C. Teller *

ABSTRACT: Proton binding changes occurring upon dilution of α-chymotrypsin solution were measured with a specially designed pH recording system. Correlation of these proton binding changes with disruption of α-chymotrypsin dimers during dilution shows that the ionization of certain groups on the enzyme is perturbed in the dimeric state. The pH dependence of the proton binding changes agrees closely with predictions made by K. C. Aune and S. N. Timasheff [(1971), *Biochemistry 10,* 1609] on the basis of the observed pH dependence of α-chymotrypsin dimerization. The observed proton binding changes during dimerization are directly predicted by a model in which the pH dependence of dimerization arises from competition between protons and other chymotrypsin molecules for the same sites on a chymotrypsin molecule.

Self-association reactions of proteins are routinely observed to be dependent on pH. Self-association of insulin (Steiner, 1952), lysozyme (Sophianopolis and van Holde, 1964), α-chymotrypsin (Steiner, 1954; Jandorf *et al.*, 1955; Aune and Timasheff, 1971; Aune *et al.*, 1971), β-lactoglobulin (Townsend *et al.*, 1960), and fibrin (Sturtevant *et al.*, 1955; Endres *et al.*, 1966; Endres and Scheraga, 1966) have all been shown to be pH dependent. This pH dependence implies that proteins preferentially associate when in a given protonated state. Since proton binding to the protein and protein self-association reactions involve the same equilibrating species, pH dependent protein self-association reactions may be examples of competing equilbria, as described by Wyman's "linked function" theory (Wyman, 1964). An alternative explanation for the pH dependence of reversible self-association of proteins can be based upon a combination of the electrostatic repulsion arising from two charged spheres (Verwey and Overbeek, 1948) together with the attraction of specific electrostatic groups (Timasheff, 1969).

Wyman's theory (1964) states that the change in the log of the equilibrium constant resulting from a change in the log of the activity of the ligand is equal to the difference in ligand binding by the two states described by the equilibrium constant. In the case of a dimerization constant which is a function of pH, the theory predicts that the binding of protons changes during the association reaction, resulting in

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a change in the pH of the surrounding medium. One does not expect such pH changes in the charged sphere-specific group analysis as presented by Timasheff (1969), but K. C. Aune (personal communication) has suggested that proton binding changes during dimerization are predicted by an extended version of this model. Timasheff's model included perturbation of the pK of a specific group on the monomer by the overall charge on the same monomer. Aune has suggested a similar perturbation might occur upon approach of the other monomer in dimer formation, leading to a pK shift during dimerization in this model, too. These theories were developed for the case of limited self-association such as the dimerization which occurs with α-chymotrypsin. For unlimited polymerization such as fibrin clot formation, Sturtevant *et al.* (1955) have developed an analysis which is basically different (see also Endres and Scheraga, 1966).

α-Chymotrypsin dimerization in the low pH region has been analyzed using several theories (Steiner, 1954; Timasheff, 1969; Aune and Timasheff, 1971). Most recently, Aune and Timasheff (1971) examined the pH dependence on the basis of Wyman's equation and were able to fit the observed dimerization constant as a function of pH with this theory. Timasheff (1969) has also fit the pH dependence of chymotrypsin dimerization with the charged sphere-specific group theory and we have confirmed this calculation with our data. Since both theories lead by curve fitting to reasonable explanations of the observed association behavior, additional information is needed to determine which is correct.

Changes in the position of the terminal carboxyl group of tyrosine-146 in crystalline α-chymotrypsin dimers have been observed when the pH of the medium is raised from 4.2 to 6.7 (Vandlen and Tulinsky, 1973). Tyrosine-146 is believed to be necessary for both crystal and free solution dimer formation by α-chymotrypsin (Horbett and Teller, 1973) so that changes in the position of the group with pH may be related to the initial events in the dissociation of the dimeric molecule caused by a pH change. Thus, Vandlen and Tulinsky (1973) suggested a refinement of Aune and Timasheff's (1971) mechanism for explaining the pH dependence of α-chymotrypsin dimerization. The refined mechanism assigns different pK values to the ionizable groups involved but retains the idea of shifts in pK between the monomeric and dimeric states. However, the presence of a tightly bound sulfate ion near tyrosine-146 in the crystal dimer which apparently bridges the monomers, and the high ionic strength medium in which the crystals are kept, suggest some caution in using the crystal dimer results to explain the pH dependence of the free solution dimerization. Furthermore, the crystal results do not provide evidence on the postulated pK shift between dimer and monomer, the subject to which this work was addressed.

Differences in proton binding between chymotrypsin dimers and monomers were directly measured by recording pH changes resulting from dilution of concentrated chymotrypsin solutions. We have found that the results are completely in accord with the linked function theory of Aune and Timasheff (1971) and provide direct evidence of the importance of proton binding to self-association of α-chymotrypsin.

Materials and Methods

The α-chymotrypsin used throughout these studies was Worthington three times crystallized (lot numbers CDI-6144-5 and CDI-OCA). Diisopropyl fluorophosphate was purchased from Merck. Dip[1]-chymotrypsin was prepared according to Laskowski (1955) and crystallized three times. Chymotrypsinogen (five times recrystallized, lot CGC IBA) was a Worthington product.

Sedimentation equilibrium studies were performed in Beckman Model E ultracentrifuge equipped with RTIC unit using Rayleigh interference optics. The data were analyzed with computer programs developed in this laboratory. Molecular weight data were used to determine the equilibrium constants by the methods presented by Teller *et al.* (1969) and Hoagland and Teller (1969). For all of the pH studies in the ultracentrifuge the solvent used was 0.20 M KCl–0.01 M potassium acetate. The pH of the solutions was adjusted prior to the dialysis and checked again at the end of the dialysis period.

Plates were read on a modified Nikon Model 6C Microcomparator (Teller, 1967). The partial specific volume of α-chymotrypsin was taken to be 0.736 ml/g at 20° (Wilcox *et al.*, 1957). Partial specific volumes were corrected for temperature as -5×10^{-4} ml/(deg g) (Svedberg and Pedersen, 1940). Density of the potassium chloride solution was measured at 20° to be 1.00763 g/ml.

Proton Release/Uptake Measurements on α-Chymotrypsin. The uptake or release of protons by α-chymotrypsin upon dilution was measured using a specially designed pH measurement system. The components of this system were a Radiometer GK2302C pH electrode, a Radiometer TTlc pH meter, a Radiometer pHA630T scale expander, a Textronix 561A oscilloscope equipped with a Textronix 3A9 differential amplifier a Varian Associates G-14 recorder, and a Sola CVS constant voltage transformer used to provide power to the pH meter and scale expander.

A 10-kΩ potentiometer was used as a voltage divider between the output of the differential amplifier and the input of the recorder and adjusted so that a 0.1 pH change would cause full scale recorder deflection when the recorder was on the 1 V scale and the differential amplifier was adjusted to approximately 1.5 mV/division sensitivity. The recorded signal was obtained from the terminals of the scale expander indicator needle drive. This signal was divided and put into both of the channels of the differential amplifier. One channel was set to "AC" and the other to "DC" so that common mode AC signals were largely rejected before the DC signal reached the recorder *via* the output lead of the differential amplifier. The recorder deflection was found to be linear with scale expander needle deflection over a range of at least 0.12 pH.

Noise and drift levels of this system were such that short term pH changes (approximately 1 sec long) as small as 0.001 pH could be readily detected and could be measured with a precision of $\pm$0.0003 pH.

A 20-ml hollow-walled reaction cup was kept at 20 $\pm$ 0.1° with a Haake Type F thermostated water circulator. Use of a vertical shaft plastic paddle stirrer (Radiometer) driven by a Radiometer M-11 motor greatly reduced apparent fluctuations encountered with magnetic stirring bars, provided the paddle was appropriately positioned relative to the electrode to minimize other stirring artifacts.

Protein solutions (30–60 mg/ml) were made using lot CDI-OCA α-chymotrypsin from Worthington Biochemical Corp. Chymotrypsin dissolved in 0.2 M KCl was titrated to the desired pH by slow addition of 1.0 and 0.1 N HCl and

[1] Abbreviation used is: Dip, diisopropylphosphoryl.

NaOH to the rapidly stirred solution. The solution was dialyzed overnight at 5° against 500 ml of 0.2 M KCl at the same pH as the protein. The protein was then removed to freshly washed glass centrifuge tubes, and these tubes were stoppered and kept in a 20° water bath during use.

The dilution solvent was made by titrating 0.2 M KCl with 1 N HCl or NaOH to within 0.02 pH of protein solution's pH. This solvent was kept in a glass-stoppered bottle in the same 20° water bath as the concentrated protein solution.

The actual dilution experiments were performed by adding 0.04–1.0 ml of concentrated protein solution to 10.0 ml of dilution solvent and recording the pH changes ensuing. Additions of protein between 0.1 and 0.5 ml were done in successive 0.1-ml increments to accumulate repetitive data. Known amounts of HCl or NaOH in 0.01–0.1-ml volumes were then added and pH changes recorded. The pure dilution solvent was titrated in this manner in a separate experiment. At least four titrant additions were made to each solution. The pH of the stock concentrated protein was recorded after all other measurements. After this exposure to the concentrated protein solutions, the electrode was immersed in slowly stirred 0.1 N HCl at room temperature overnight, since otherwise its response characteristics deteriorated markedly.

Linderstrøm-Lang micropipets were mechanically driven to deliver protein solutions and Gilson micrometer burets were used to deliver HCl or NaOH to the diluted solutions or to titrate the dilution solvent. HCl and NaOH were made using boiled distilled water and J. T. Baker "Dilut-It" analytical concentrates.

Recordings of several minutes duration preceded and followed each addition to the reaction vessel. Visually smoothed straight lines drawn through these recordings were extrapolated to the point of addition and the actual pH change, in chart units, was measured there. Errors due to instability in the recordings, usually due to long term pH drift or ocassional stirring artifacts during additions, were believed to be largely avoided by this procedure.

Theory and Calculations. Let the change in the moles of H^+ bound by a mole of dimer upon monomerization be denoted by $-\Delta\nu_{H^+}$.[2] In order to measure $\Delta\nu_{H^+}$, it is necessary to correct for residual pH differences between the concentrated protein solution and the dilution solvent and for the titration curves of protein and solvent. The procedure used assumed linearity of the titration curves of both protein and solvent over the small pH changes involved in these experiments (0.1 pH unit maximum). The proton release from the dimer is given by the relation

$$\Delta\nu_{H^+} = (\Delta H^+_{obsd} - \Delta H^+_{CF})/\Delta D \quad (1)$$

where ΔH^+_{obsd} is the observed change in binding, in moles of proton, ΔH^+_{CF} is the apparent change in binding (in moles proton) due to initial pH differences, solvent and protein titration, and dilution, and ΔD is the change in the number of moles of dimer between concentrated and diluted solutions.

If we let V_p denote the number of liters of protein added to the reaction cup and V_s the number of liters of dilution solvent added to it, then

$$\Delta D = V_p[C_2]^I - (V_p + V_s)[C_2]^F \quad (2)$$

[2] This sign convention was used to make our notation for the dissociation reaction consistent with that used by Aune and Timasheff (1971) for the association reaction.

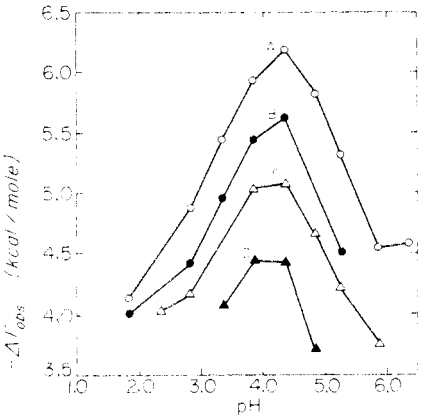

FIGURE 1: pH and temperature dependence of the free energy of dimerization of α-chymotrypsin and Dip-chymotrypsin. (A) α-chymotrypsin at 20°; (B) α-chymotrypsin at 5°; (C) Dip-chymotrypsin at 20°; (D) Dip-chymotrypsin at 5°.

In this equation, $[C_2]$ is the molar concentration of dimer and the superscripts I and F denote initial and final values of this quantity. The values of $[C_2]$ were calculated from the protein concentration and the equilibrium constants determined for α-chymotrypsin at 20° in 0.2 M KCl–0.01 M potassium acetate determined by sedimentation equilibrium studies.

The equation used to calculate ΔH^+_{obsd} is

$$\Delta H^+_{obsd} = (\Delta pH)_{obsd}[(m_p\bar{T}_p) + (V_s\bar{T}_s)] \quad (3)$$

In this equation $(\Delta pH)_{obsd}$ is the observed change in pH (initial pH subtracted from final pH), m_p is the number of grams of protein added. $\bar{T}_s$ and $\bar{T}_p$ are the average titration factors for solvent and protein, respectively, measured at the pH of the dilution experiment. T_s for each experiment was calculated from the relation

$$T_s = (\Delta H^+/\Delta pH)/(V_s + V_T) \quad (4)$$

Here a known number of moles of HCl or NaOH (ΔH^+) in a known volume V_T (in liters) was added to a known volume of dilution solvent V_s (in liters) and the resulting change in pH (ΔpH) measured. These values were averaged to obtain $\bar{T}_s$ of eq 3.

To obtain T_p the protein solution resulting from the dilution experiment was titrated and the average titration due to solvent subtracted

$$T_p = \frac{1}{m_p}[(\Delta H^+/\Delta pH) - (V_s + V_p)\bar{T}_s] \quad (5)$$

As in the case of eq 4, several values of T_p were averaged to yield $\bar{T}_p$.

The final term of eq 1, ΔH^+_{CF}, was calculated from the relation

$$\Delta H^+_{CF} = (pH_p - pH_s)(m_p\bar{T}_p + V_p\bar{T}_s). \quad (6)$$

In this equation, pH_p was the initial pH of the protein solution and pH_s, the initial solvent pH.

The $\Delta\nu_{H^+}$ values reported at each pH are averages of at least ten experiments at that pH. Further, the pH values of these individual experiments were also averaged.

Results and Discussion

The free energies of dimerization of α-chymotrypsin and Dip-chymotrypsin were measured at two temperatures in the pH range 2–6. Figure 1 presents these results, which make clear the qualitative similarity of the pH and temperature dependence of the native and inhibited enzyme, even

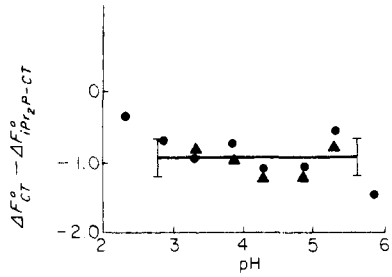

FIGURE 2: pH dependence of the difference in free energy of dimerization between α-chymotrypsin and Dip-chymotrypsin. Circles represent the difference at 20°. Triangles represent the difference at 5°. The horizontal line indicates the average difference for all the data. The standard deviation of this average is indicated by the vertical bars, which are two standard deviations in length.

though the Dip derivative dimerizes with a quantitatively lower free energy. This fact is more clearly seen in Figure 2, where the difference in free energy of dimerization between Dip-chymotrypsin and native enzyme is plotted. Since this difference in free energies between the native enzyme and its inhibited derivative show no variation as a function of pH we may conclude that the lower degree of association of the Dip derivative is due to steric hindrance, rather than any shift in pK of the groups. Such pK shifts have been proposed to explain the reduced association observed for several other chymotrypsin derivatives by Neet and Brydon (1970).

The results in Figure 2 also indicate it would be possible to combine all of the data into single averages at each pH in order to remove experimental uncertainties. Figure 3 shows the results of normalizing the data of Figure 1 to the results of α-chymotrypsin at 20°.[3] Comparison of these results with those reported previously by Aune and Timasheff (1971) is complicated by the use of higher ionic strength in the experiments reported here. Since Aune and Timasheff also showed that the effect of ionic strength on the free energy of dimerization varied with pH, a different correction factor is necessary at each pH. To arrive at the corrected values, we used the data in their Figures 2 and 3 to construct a plot of $\Delta \ln K/\Delta \ln a$ *vs.* pH, which we found to be linear over the pH range utilized. Aune and Timasheff's data were found to fit the empirical equation

$$\frac{\Delta \ln K}{\Delta \ln a} = -0.415\text{pH} + 2.73 \qquad (7)$$

K is the dimerization constant and a is the activity of the salt in the solvent. This equation was used to correct the lower ionic strength data of Aune and Timasheff to our conditions. The results of the two studies were then found to be exactly superimposable (see Figure 3).

Comparison of the pH dependence for dimerization of chymotrypsin observed here to the predicted behavior based on the linked function model of Aune and Timasheff is also made in Figure 3, where the solid line was calculated using the pK values which Aune and Timasheff found to fit their data (see Table I). At pH's less than 3 and greater than 5, small deviations of the data from the predicted values occur (see Figure 3). Since the entire set of data can be fit exactly by the charged sphere model presented by Timasheff in 1969, there is some cause to consider these minor deviations

[3] For each of the four curves of Figure 1, the ΔF values were averaged. The difference of the average of a curve from the average of α-chymotrypsin at 20° was then subtracted from all individual ΔF values, resulting in a net shift up of the entire curve.

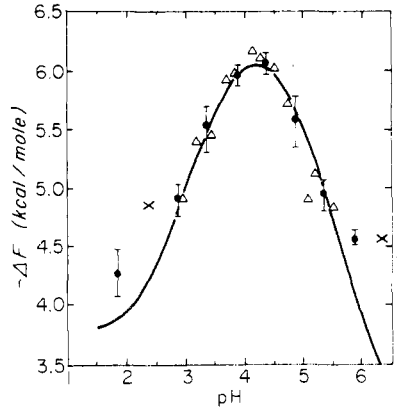

FIGURE 3: pH dependence of dimerization of α-chymotrypsin at 20°, 0.2 M ionic strength. The circles represent the normalized average of all the data in Figure 1 (see text). X indicates points for which only one observation was available. The triangles represent the data of Aune and Timasheff (1971) after correction to 0.2 M ionic strength. The smooth curve was calculated from the model of Aune and Timasheff (1971).

from the pK model as important. However, we believe at the present time that the deviations at low pH regions are due to various impurities in the chymotrypsin preparations (Horbett, 1970) and the deviations at high pH are due to separate phenomena; *viz.*, a different stoichiometry of self-association (Rao and Kegeles, 1958). In any case, either method of analyzing the data gives reasonable agreement with the observed results.

The linked function model of Aune and Timasheff (1971) directly predicts proton binding changes during dilution of α-chymotrypsin solutions, but Aune's (personal communication) extension of the charged sphere-specific group theory of Timasheff (1969) also predicts these changes. However, the pH dependence of proton binding changes predicted by the two models is quite different, since the pK perturbations causing the proton binding changes arise by different mechanisms in the two models. [A third mechanism for proton release during polymerization, based on intermolecular hydrogen bonding, was presented by Sturtevant *et al.* (1955).] Thus, a method of distinguishing the linked function model (Aune and Timasheff, 1971) from the charged sphere model (Timasheff, 1969) is to directly measure the change in hydrogen ion binding upon dilution of α-chymotrypsin at various pH values. Figure 4 presents the results of measurements of the change in hydrogen ion binding to chymotrypsin as a function of pH. The solid line is the curve predicted for $\Delta\nu_{H^+}$ *vs.* pH using the pK values of Table I, which were taken directly from Aune and Timasheff (1971). The close agreement of this theoretical curve with the observed results is clear from this figure.

The theoretical curve for $\Delta\nu_{H^+}$ *vs.* pH expected from the charged sphere-specific group analysis cannot be predicted very well since the electrostatic work function needed for this calculation is not known for the case at hand, namely the close approach of a charged monomer to another monomer carrying the group to be perturbed. The general shape of the curve can be deduced from the equation (Timasheff, 1969)

$$K_i = K_i^0 e^{2wZ} \qquad (8)$$

which relates the ionization constant for group i, K_i, to the intrinsic ionization constant for that group, K_i^0, to the electrostatic work function w, and to the overall charge on the

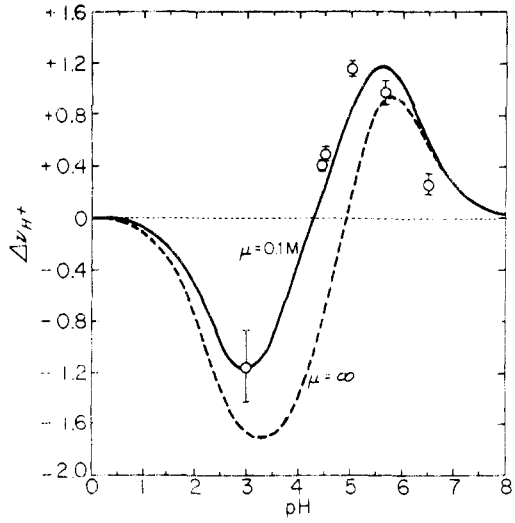

FIGURE 4: Proton binding changes in α-chymotrypsin dimerization. The points represent the observed change in the number of protons bound by α-chymotrypsin upon dimerization, in moles of proton per mole of dimer. The vertical bars through the points are 2 standard deviations long. The curves were calculated using the model of Aune and Timasheff (1971) with either the pK values found to fit data at 0.1 M ionic strength (solid curve, μ = 0.1 M) or the pK values calculated to exist in the absence of charge effects (dotted curve, $\mu = \infty$).

monomer, Z. Since Z is zero at the isoelectric pH of the protein, and always positive below the isoelectric point, this equation predicts no perturbation of the ionization of the group by approach of another monomer at the isoelectric pH, and a uniformly increasing perturbation below the isoelectric pH. Thus, this analysis predicts $\Delta\nu_{H^+}$ would be zero at the isoelectric pH and would increase uniformly in magnitude to either side of the isoelectric pH. This prediction is clearly very different from the observed $\Delta\nu_{H^+}$ *vs.* pH data in Figure 4, which are seen to rise and then fall between pH 7 and 4, and to actually reverse sign below pH 4. The isoelectric pH of α-chymotrypsin (8.5) is well above the pH range of these changes (Rao and Kegeles, 1958).

Parallel dilution experiments with chymotrypsinogen were also done at pH 3.0 and 5.4, near where the maximum proton binding changes were observed for chymotrypsin (see Figure 4). The proton binding changes occurring for the same dilution for the two proteins were compared assuming both proteins were completely monomeric, since chymotrypsinogen dimerizes very little, if at all (Miller *et al.*, 1971). The proton binding changes per mole of chymotrypsinogen averaged 14 and 13%, respectively, of those for chymotrypsin. These values are negligible within experimental error. It appears the proton release upon dilution of chymotrypsin is not due to some nonspecific process such as any protein might display, but is due to its dimerization.

The $\Delta\nu_{H^+}$ *vs.* pH data thus appear to agree well with theory and seem to be specific to the dimerization process. However, a more critical appraisal of these data reveals that the observed proton release data are systematically displaced to the low pH side of the predicted curve (see Figure 4). We believe this is probably due to the use of 0.2 M ionic strength in the proton release experiment, while the pK's used to obtain the predicted proton release curve were obtained by fitting dimerization data taken at 0.1 M ionic strength (Aune and Timasheff, 1971). The fact that the 0.1M ionic strength based pK's do seem to predict the 0.2 M ionic strength dimerization data (see Figure 3) while appearing to be slightly in error in predicting the 0.2 M ionic strength proton release data is probably at least partially explained by the relative insensitivity of the ΔF *vs.* pH data. These data are in reality a log–log plot (RT log K *vs.* $-$log [H$^+$]) in comparison to the proton release curve which directly measures the effects of any shifts in pK of the dimerizing molecules ($\Delta\nu_{H^+}$ *vs.* $-$log [H$^+$]). Other uncertainties arise from the use of four parameters (the pK values) to fit data over a relatively small pH range. This discussion exemplifies what we believe to be a fundamental advantage of proton release measurements in pH dependent processes, namely the sensitive demonstration of involvement of pK shifts during the process. We conclude, therefore, that the hypothesis of Aune and Timasheff is the correct explanation of the pH dependence of chymotrypsin dimerization at low pH.

TABLE I: pK's Used for $\Delta\nu_{H^+}$ *vs.* pH Curves (Figure 4).[a]

Ionic Strength (M)	p$K_{1,m}$	p$K_{1,d}$	p$K_{2,m}$	p$K_{2,d}$
0.1	5.0	6.2	3.6	2.4
∞	5.2	6.2	4.5	2.2

[a] The numerical subscript indicates the group to which the pK refers, the m or d subscript refers to monomer or dimer, respectively.

The linked function theory of Wyman (1964) has been experimentally verified in these studies on chymotrypsin dimerization but it would appear that similar studies on other proteins may be difficult. The high molar solubility and simple stoichiometry of the chymotrypsin system at low pH make it ideal for proton release measurements. In this case, closely spaced, highly accurate data were not necessary to demonstrate the fit to the predicted curve. Even this simple system required four parameters to fit it, however, so it is clear that systems involving more than a few ionizable groups in the association reaction, or in which the groups are involved in multiple modes of association (dimer, trimer, etc.), may be extremely difficult to characterize.

In spite of these potential difficulties, the possibility that all self-associating systems involve a shift in pK of ionizable groups is intriguing and does appear worth pursuing. It seems reasonable to expect that the dissociation behavior of ionizable groups may be perturbed by the change in the electrostatic environment which accompanies the close approach of charged monomers in an association reaction. Furthermore, several protein–protein interactions are believed to involve their (charged) terminal amino acids. This is the case for α-chymotrypsin (Horbett and Teller, 1973), hemoglobin (Perutz, 1970), and lactic dehydrogenase (Adams *et al.*, 1970). Finally, it may be speculated that an easy way to convert an enzyme from a single polypeptide chain to an oligomer would be the addition of a sticky "tail" on one or the other terminus. The proof of the existence of such a possibly general mechanism for oligomerization would constitute a fundamental contribution to our knowledge of protein chemistry, and it is in this light that further proton release measurements on other systems may be useful.

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Binding of Peptides to Elastase: Implications for the Mechanism of Substrate Hydrolysis†

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ABSTRACT: Equilibrium constants for the binding of several peptides to elastase have been measured and the free energies for enzyme-peptide complex formation calculated. The variation in binding energy with structure of the peptide points to several important aspects of the catalytic mechanism of the enzyme. The increased rate of hydrolysis noted for long peptides can now be shown to be due to a specific destabilization of the scissile amide bond in Michaelis complexes of the enzyme with long peptides. Stabilization of the transition state complex with long peptides exerts a smaller, but still appreciable, effect on the rate of substrate hydrolysis. Replacement of the substrate's planar scissile bond with tetrahedral groups results in at most a 2 kcal/mol increase in enzyme-peptide affinity. Strain in the enzyme-substrate complex is therefore unlikely to be the most important factor contributing to catalysis of hydrolysis. Large increases in enzyme-peptide binding, about 5 kcal/mol, are seen with peptides which form what would normally be considered a rather labile hemiacetal bond with the enzyme. The extra stability of these transition state analog complexes coincides quite well with that expected if covalent bond formation between the peptide and the enzyme involved no loss of entropy. Much of the catalytic power of elastase may therefore be due to nothing more complicated than its conversion of an entropically unfavorable bimolecular reaction to a unimolecular reaction by virtue of the formation of an enzyme-substrate complex.

Many enzymes which modify biological macromolecules are known to form significant enzyme-substrate contacts at points distant from the immediate site of chemical modification. Among the best characterized of these enzymes are those which catalyze the hydrolysis of polysaccharides and proteins. Extensive studies of enzyme-substrate interactions have been reported for lysozyme (Imoto *et al.,* 1972), pepsin (Fruton, 1971), carboxypeptidase A (Abramowitz *et al.,* 1967), papain (Schechter and Berger, 1968), subtilisin (Morihara *et al.* 1970; Kraut *et al.,* 1972), and chymotrypsin (Segal *et al.,* 1971). The forces involved in these interactions are rather easily studied through systematically varying the substrate. These systems have therefore been

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