

- Brunori, M., Alfson, A., Saggese, U., Antonini, E., and Wyman, J. (1968a), *J. Biol. Chem.* **243**, 2950.
- Brunori, M., Amiconi, G., Antonini, E., Wyman, J., Zito, R., and Rossi-Fanelli, A. (1968b), *Biochim. Biophys. Acta* **154**, 315.
- Brunori, M., Antonini, E., Wyman, J., Zito, R., Taylor, J. F., and Rossi-Fanelli, A. (1964), *J. Biol. Chem.* **239**, 2340.
- Brunori, M., Taylor, J. F., Antonini, E., and Wyman, J. (1969), *Biochemistry* **8**, 2880.
- Brunori, M., Taylor, J. F., Antonini, E., Wyman, J., and Rossi-Fanelli, A. (1967), *J. Biol. Chem.* **242**, 2295.
- Brunori, M., Wyman, J., Antonini, E., and Rossi-Fanelli, A. (1965), *J. Biol. Chem.* **240**, 3317.
- Clark, W. M. (1960), *Oxidation-Reduction Potentials of Organic Systems*, Baltimore, Md., Williams & Wilkins.
- Coryell, C. D., Stitt, K. F., and Pauling, L. (1937), *J. Amer. Chem. Soc.* **59**, 633.
- Dickerson, R. E., Kopka, M. L., Weinzierl, J. E., Varnum, J. C., Eisenberg, D., and Margoliash, E. (1967), *J. Biol. Chem.* **242**, 3015.
- Harbury, H. A. (1957), *J. Biol. Chem.* **225**, 1000.
- Harbury, H. A., and Loach, P. A. (1960), *J. Biol. Chem.* **235**, 3646.
- Huber, R., Epp, O., and Formanek, H. (1969), *J. Mol. Biol.* **42**, 591.
- Huber, R., Epp, O., and Formanek, H. (1970), *J. Mol. Biol.* **52**, 349.
- Huber, R., Formanek, H., Braun, V., Braunitzer, G., and Hoppe, W. (1964), *Z. Elektrochem.* **68**, 818.
- Keilin, J. (1966), in *Hemes and Hemoproteins*, Chance, B., Estabrook, R., and Yonetani, T., Ed., New York, N. Y., Academic Press, p 173.
- Kendrew, J. C. (1962), *Brookhaven Symp. Biol.* **15**, 216.
- Kunkel, H. G. (1954), *Methods Biochem. Anal.* **1**, 141.
- Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. G. G. (1968), *Nature (London)* **219**, 131.
- Rodkey, F. L., and Ball, E. G. (1950), *J. Biol. Chem.* **182**, 17.
- Rossi-Fanelli, A. (1949), in *Haemoglobin*, Roughton, F. J. W., and Kendrew, J. C., Ed., London, Butterworths, p 115.
- Rossi-Fanelli, A., and Antonini, E. (1957), *Biokhimiya* **22**, 336.
- Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1958), *Biochim. Biophys. Acta* **30**, 608.
- Russel, A. D., and Pauling, L. (1939), *Proc. Nat. Acad. Sci. U. S.* **25**, 517.
- Taylor, J. F., and Morgan, V. E. (1942), *J. Biol. Chem.* **144**, 15.
- Tentori, L., Vivaldi, G., Carta, S., Antonini, E., Brunori, M. (1968), *Nature (London)* **219**, 487.

## Dimerization of $\alpha$ -Chymotrypsin.

### I. pH Dependence in the Acid Region\*

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**ABSTRACT:** The dimerization equilibrium constant of  $\alpha$ -chymotrypsin was determined by the method of sedimentation equilibrium as a function of pH. The pH dependence was interpreted in terms of short-range electrostatic interactions

between two pairs of identical ionizable groups in the enzyme. From a consideration of the known crystal structure, these groups were identified as the imidazole ring of histidine 57 and the  $\alpha$ -carboxyl of tyrosine 146.

In recent years, it has become evident that the association between protein molecules plays an important role in the control of enzyme activity. Such associations affect catalytic rates by altering binding constants for substrates and products. The allosteric models of Koshland *et al.* (1966) and of Monod *et al.* (1963) discuss the linkage of subunit interactions with enzyme activity. The specific interactions which would supply the free energy needed to affect the activity can be

expected to differ greatly for various systems. Therefore, it is highly desirable to specify the types of interactions which might be operative in any given system, rather than to generalize. The present study on the dimerization of  $\alpha$ -chymotrypsin is directed toward that goal.

The self-association of  $\alpha$ -chymotrypsin has been the subject of a number of studies. About 20 years ago, Schwert (1949) and Schwert and Kaufman (1951) observed that the sedimentation coefficient of the protease, bovine pancreatic  $\alpha$ -chymotrypsin was concentration and pH dependent, even though the sedimentation pattern was characterized by only a single symmetrical schlieren peak. They suggested that this was the result of a monomer-dimer equilibrium, but they considered that the reaction was complex, because the concentration dependence of  $s_{20,w}$  could not be described in terms of a simple monomer-dimer equilibrium constant. Later, Steiner (1954) investigated the aggregation reaction by means of light scattering, showing that the reaction was in fact a monomer-dimer equilibrium and that the association was enhanced at pH 4.4 by an increase in ionic strength. Egan *et al.* (1957) reported that the sedimentation coefficient of  $\alpha$ -chymotrypsin

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reached a maximum in the region of pH 4.0. Furthermore, they observed that photooxidation of a single histidine caused the disappearance of any tendency to associate. From their results, they suggested that a charged imidazole group, probably that in the active site, and a carboxylate ion were involved in the dimerization reaction.

From the sedimentation velocity data of Egan *et al.* (1957), Timasheff (1969) calculated the equilibrium constant for the dimerization of  $\alpha$ -chymotrypsin according to the method of Gilbert (1955). Employing an electrostatic model in which screened Coulombic potentials were used for specific charged group interactions and a Verwey-Overbeek (1948) potential for the general electrostatic repulsion between two charged spheres, the data in the pH range between 2.7 and 7.2 could be fitted to a model containing a pair of identical anionic-cationic attractions and an anionic-anionic repulsion located about a dyad axis of symmetry between the two molecules.

Above pH 6,  $\alpha$ -chymotrypsin undergoes aggregation of a higher order than monomer-dimer (Gilbert, 1955; Massey *et al.*, 1955; Neurath and Dreyer, 1955; Tinoco, 1957; Rao and Kegeles, 1958; Bethune and Kegeles, 1961). As a result, the present study has been limited to a characterization of the dimerization reaction which occurs below that pH. The pH dependence of the dimerization equilibrium was determined precisely by the method of sedimentation equilibrium, and the results were interpreted by a treatment in which the  $pK$ 's of the groups involved were determined directly from the association data. The conclusions were compared with recently available information on the structure of  $\alpha$ -chymotrypsin in the crystal. The dependence of this association on temperature and ionic type and concentration, reported in the following paper (Aune *et al.*, 1971), has given further insight into the nature of forces involved in this system and to the importance of solvent participation in the intermolecular interactions.

## Materials and Methods

Worthington three-times-crystallized  $\alpha$ -chymotrypsin, lots CDI 8LK, CDI 8JF, and CDI 6JA, were used without further purification, since no significant contaminant was found either by chromatography on G-75 Sephadex in 0.001 M HCl or from the schlieren patterns in sedimentation velocity experiments. Other chemicals used in this study were standard reagent grade.

The pH of the solution was measured at room temperature ( $\sim 25^\circ$ ) on a Radiometer Model 4 pH meter equipped with a Radiometer GK 2321 combination glass electrode, which was always standardized with Fisher pH 4.00 standard buffer just prior to use.

The concentrations of  $\alpha$ -chymotrypsin in all solutions were determined spectrophotometrically on a Cary 16 spectrophotometer using an absorptivity value of 20.3 dl/(g cm) at 280 nm, determined by the method of dry weight at  $100^\circ$  in a vacuum oven. The partial specific volume was taken to be 0.736 at  $25^\circ$  (Schwert and Kaufman, 1951).

The concentrations of  $\alpha$ -chymotrypsin at different radial positions at sedimentation equilibrium were determined from a calibration of fringe displacement by protein at a known concentration, using a double-sector, capillary-type synthetic boundary cell. Assuming that the refractive index increment,  $dn/dc$ , is constant, the protein concentration,  $C$ , is a linear function of  $f$ , the fringe displacement (relative to solvent).

$$C = kf \quad (1)$$

The value of  $k$  was found to be 0.000882 g/(l.  $\mu$ ) which corresponds to a value of 0.000186 l./g for  $dn/dc$  at 546 nm, in excellent agreement with 0.000187 l./g, found by Sarfare *et al.* (1966).

The sedimentation experiments were carried out at  $25^\circ$  in a Spinco Model E analytical ultracentrifuge equipped with schlieren and Rayleigh interference optics, electronic speed control, and RTIC temperature control unit. In sedimentation equilibrium experiments, epon-filled aluminum or Kel-F coated aluminum double-sector centerpieces with sapphire windows were used. The sedimentation equilibrium experimental results, being interference patterns, were recorded on Kodak III-G spectroscopic plates by exposure to the light of an AH-6 lamp filtered by a Baird Atomic B-9 interference filter or by the combination of a Kodak wratten filter 77A and a polarizing lens. The fine interference mask was used to provide a wide envelope of fringes. Because of this, as well as of the use of the adjustable slit at a very narrow opening, common exposure times were of the order of 30 min. A shutter was employed as suggested by D. C. Teller (personal communication) to allow a white light exposure of air reference fringes; these facilitate optimum alignment of the plates on a Nikon 6C microcomparator. The method of high-speed equilibrium, described by Yphantis (1964), was modified to allow for a broader concentration distribution. The appropriate combinations of rotor speed, column height, and protein concentration always provided for a meniscus concentration of less than 8  $\mu$  displacement. Water blank pictures were taken, but generally there was no need to make corrections, since the maximum corrections were less than 15  $\mu$  from the meniscus to the bottom of the cell.

**Data Reduction.** Three white fringes were read and positions were averaged from near the meniscus to the base of the cell every 100  $\mu$ , beginning with a vertical displacement of approximately 30–40  $\mu$  relative to the meniscus displacement. For some of the later experiments the plate readings were entered directly into the keyboard of a Wang 370 programmable electronic calculator equipped with three card readers, 64 storage registers, and a teletype input-output device. The vertical positions were printed out while the average was computed and, if the average deviated from the central value by more than 5  $\mu$ , the system indicated that the vertical displacements were to be reread. This procedure greatly reduced the time involved in plate reading; it also eliminated a certain amount of bias and erratic data points. Another less desirable method used in reducing the amount of noise in the data was to input the averaged fringe displacements on paper tape into the Wang 370 system, where a sliding five-point parabolic fit was calculated. The calculated displacements continually replaced the experimental data to provide a more continuous curve; this method, however, is certainly not as sophisticated as the smoothing processes devised by Teller *et al.* (1969) and Roark and Yphantis (1969) and caution was taken not to introduce artifacts. Corrections due to this method generally were less than 5  $\mu$  from the raw averaged data.

The comparator raw data (horizontal positions and vertical displacements) were punched on paper tape and read into the Wang 370 system controlled by programs that have been developed in this laboratory. The output is a table of horizontal comparator positions,  $X$ , fringe displacements,  $f$ , radial distances squared,  $r^2$ , natural logarithms of the fringe displacements,  $\ln f$ , the point number-average molecular weights,  $M_n(r)$ , and the point weight-average molecular weights,  $M_w(r)$ . Concomitantly a paper tape is generated containing the values of  $\ln f$ ,  $M_n(r)$ , and  $M_w(r)$ . This tape is

then passed through another program which computes the dimerization equilibrium constants at each point from both the number-average and weight-average molecular weights and known protein concentration.

The apparent number-average molecular weight as a function of radial distance,  $M_n(r)$ , is obtained through the relationship

$$M_n(r) = \frac{f}{f_a/M_n(a) + \frac{(1 - \bar{v}\rho)\omega^2}{2RT} \int_a^r f dr^2} \quad (2)$$

where  $f_a$  and  $M_n(a)$  are the fringe displacement and number-average molecular weight at the meniscus,  $\bar{v}$  is the partial specific volume of the protein,  $\rho$  is the density of the solution,  $\omega^2$  is the square of the angular velocity,  $R$  is the gas constant, and  $T$  is the absolute temperature. The integration constant,  $f_a/M_n(a)$ , is computed in the following manner. At the first data point where there is a displacement relative to the meniscus,  $f_1$ , of about 50  $\mu$ , a trial value for the weight-average molecular weight,  $M_{w(est)}$ , is assumed. The concentration at the meniscus is then estimated from the equation

$$\ln f_{a(approx)} \cong \ln f_1 - \frac{(1 - \bar{v}\rho)\omega^2}{2RT} (r_1^2 - r_a^2) M_{w(est)} \quad (3)$$

Assuming that

$$\frac{f_a}{M_n(a)} \cong \frac{f_{a(approx)}}{M_{w(est)}} \quad (4)$$

the number-average molecular weight is then computed at higher concentrations according to eq 2 with the integral being approximated by the expression

$$\int_a^r f dr^2 \cong \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{(f_1 - f_{a(approx)})}{M_{w(est)}} + \sum_{r=r_1}^r \tilde{f}_i \Delta r_i^2 \quad (5)$$

where  $f_1$  and  $r_1$  represent the first experimental fringe displacement and radial distance. The net effect of this type of analysis is to anchor the number-average molecular weight distribution at a low concentration to allow its more accurate evaluation at higher concentrations.

The apparent weight-average molecular weight is given by the equation

$$M_w(r) = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \left. \frac{d \ln f}{dr^2} \right|_r \quad (6)$$

The quantity  $d \ln f/dr^2$  is computed as the slope at the position of the central point of five points either from the best average straight line through the five points or the least-squares straight line through the five points. The latter method was used for most of the data reported here, while the former method was adopted in the later stages of the study, since it saves computation time and since no significant effect on error is exhibited, compared to the random error involved in calculating a sliding slope from five points at a time. The Z-average molecular weight was not computed from the data since a more sophisticated smoothing is required in order to obtain believable second derivatives of the data.

The method of data analysis described above is potentially subject to significant errors and should not be indiscriminately applied to unknown associating systems. Clearly, the proce-

dures outlined by Teller *et al.* (1969) give much higher precision. In the present study, this method was checked with respect to the precision desired for the proper interpretation of the results and was found to be adequate for the conditions reported here. Great caution must be exercised in applying this method to unknown systems, since much weight is placed on the estimated value of  $M_w(f_1)$  with the consequence that considerable error could occur at low protein concentrations. In such cases, more exacting procedures, such as that of Teller *et al.* (1969) should be applied.

Finally, if the second virial coefficient is zero, the point-average molecular weights are related to the dimerization equilibrium constant by the relationship

$$K = \frac{M_1}{2kf} \frac{1 - \alpha}{\alpha^2} \quad (7)$$

where  $K$  is the dimerization equilibrium constant,  $M_1$  is the monomeric molecular weight, and  $\alpha$  is the fraction of protein, on a mass basis, present as monomer. The quantity  $\alpha$  can be calculated from either the number-average or weight-average molecular weight with the expressions

$$\alpha_n = \frac{2M_1}{M_n} - 1 \quad (8)$$

$$\alpha_w = 2 - \frac{M_w}{M_1} \quad (9)$$

Thus, if the associating system is in fact a true monomer-dimer equilibrium, the values of  $\alpha_n$  and  $\alpha_w$  must be equal.

The average value of  $K$ ,  $K_l$  (where  $l$  represents  $n$  or  $w$ ), was computed through a weighted analysis from both the number-average and weight-average molecular weights according to the equations

$$K_l = \frac{\sum_{i=1}^N K_{li} W_{li}}{\sum_{i=1}^N W_{li}} \quad (10)$$

and

$$W_{li} = \frac{[4\alpha_{li}(1 - \alpha_{li})]^2}{|M_{li} - \bar{M}_{li}|} \quad (11)$$

with the sums taken over all point equilibrium constants. The coefficient of the point equilibrium constant is a weighting factor, the denominator of which can be recognized as being the same as the one used by Hoagland and Teller (1969), excluding the use of the Z-average molecular weight. The quantity,  $\bar{M}_{li}$ , when  $l$  represents  $n$ , is the number-average molecular weight calculated from the experimental value of  $M_{wi}$  assuming a monomer-dimer equilibrium, and the reverse is true when  $l$  represents  $w$ . The numerator of eq 11 is merely an empirical expression which varies from zero to one as a function of  $\alpha$  is a manner similar to the reciprocal of the error of  $K$ . The overall averages,  $\bar{K}_n$  and  $\bar{K}_w$ , were then averaged, since in all cases they were equivalent within experimental error.

## Results and Discussion

Sedimentation equilibrium experiments were carried out on  $\alpha$ -chymotrypsin between pH 2.3 and 5.5. At pH 2.3, 0.1 M

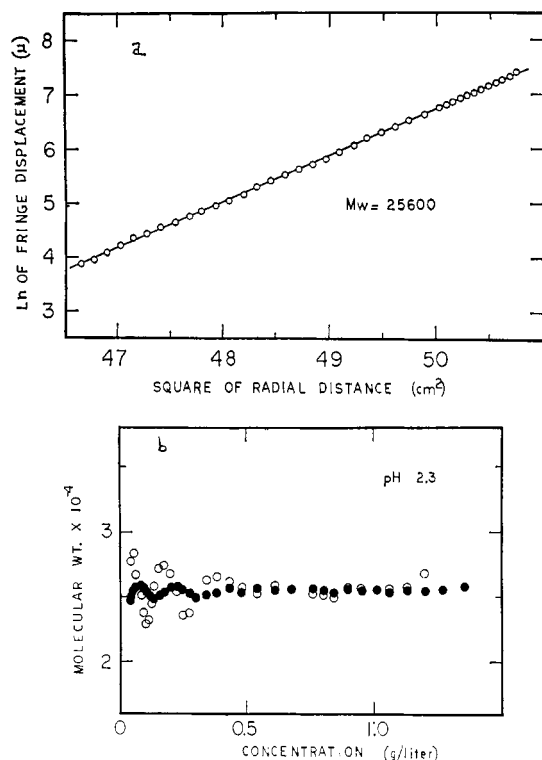


FIGURE 1: (a) Dependence of the natural logarithm of the fringe displacement (in microns) on the square of the radial distance. Conditions: 24,000 rpm, 25°, 0.1 M NaCl, pH 2.3,  $\alpha$ -chymotrypsin initial concentration, 0.36 g/l. (b) Dependence of the molecular weight averages on  $\alpha$ -chymotrypsin concentration in g/l. calculated from data in Figure 1a; filled circles represent point number-average molecular weights and open circles represent point weight-average molecular weights.

NaCl,  $\alpha$ -chymotrypsin exists in a predominantly monomeric state. This is shown in Figure 1a, in which a straight line is obtained for the plot of the natural logarithm of the fringe displacement in microns,  $\ln f$ , vs. the square of the radial distance,  $r^2$ , as predicted for a homogeneous species. The molecular weight of  $\alpha$ -chymotrypsin calculated from these data is 25,600, compared with the known molecular weight of 25,234, calculated from the amino acid sequence of Brown and Hartley (1966a,b). In Figure 1b, the calculated point number-average and weight-average molecular weights are given as a function of protein concentration. The coincidence of the number- and weight-average molecular weights without a significant trend supports the presence of a single monomeric species at these conditions. Furthermore, the absence of a trend, as well as the coincidence of the weight-average and number-average molecular weights, shows that, at an ionic strength of 0.1, the second virial coefficient is sufficiently small to be neglected, even at the acidic pH where  $\alpha$ -chymotrypsin has a net positive charge. The second virial coefficient was, therefore, taken as zero for all the other conditions of this study, since they correspond to lower net charges on the protein.

Similar plots for  $\alpha$ -chymotrypsin in 0.178 M NaCl–0.01 M acetate buffer, pH 4.1, are shown in Figure 2. A curvature is observed in the dependence of  $\ln f$  on  $r^2$  (Figure 2a). The information conveyed in this plot is not quite as useful, however, as that contained in Figure 2b, where the number-average and weight-average molecular weights are plotted vs. protein concentration. In fact, there is a certain amount of danger

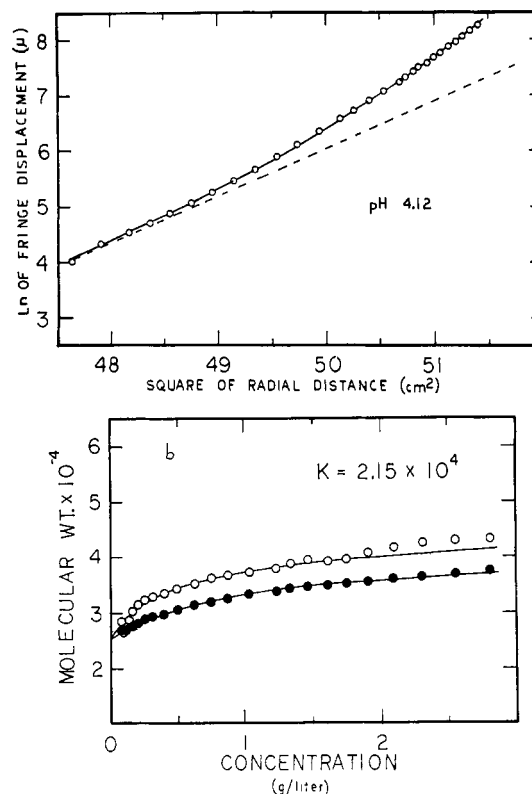


FIGURE 2: (a) Dependence of the natural logarithm of the fringe displacement (in microns) on the square of the radial distance. Conditions: 24,000 rpm, 25°, 0.178 M NaCl, 0.01 M acetate buffer, pH 4.12,  $\alpha$ -chymotrypsin concentration, 0.76 g/l. The dashed line corresponds to monomer. (b) Dependence of the molecular weight averages on  $\alpha$ -chymotrypsin concentration in g/l. calculated from data in Figure 2a; filled circles represent point number-average molecular weights and open circles represent point weight-average molecular weights.

involved in using solely plots of the type shown in Figure 2a, because neglect of data below 100  $\mu$  permits the drawing of a somewhat convincing straight line, which would correspond to a molecular weight of about 37,000 for an apparently homogeneous protein. In Figure 2b, both molecular weight averages show a trend, indicating more clearly the polydispersity of the system. The solid lines are theoretical curves calculated for a monomer–dimer association, with an association equilibrium constant of  $2.15 \times 10^4$  l./g, computed from the data in the manner described above.

The data collected for  $\alpha$ -chymotrypsin in 0.1 M NaCl, 0.01 M acetate buffers between pH 2.8 and 5.5 are summarized in Figure 3, where the natural logarithm of the dimerization equilibrium constant is shown as a function of pH. It is seen that, as the pH decreases, the association reaction is enhanced until a maximum is reached at approximately pH 4.4. Below this point, the tendency to associate falls off with further decrease in pH. Qualitatively, the same observation had been made by Egan *et al.* (1957), who found that the sedimentation coefficient went through a maximum between pH 3.8 to 4.2. Free-energy calculations (Timasheff, 1969) from Egan's data gave a maximum at approximately pH 4.0.

The pH dependence of the association could be the result of several factors. These will be examined in turn. There are two types of charge interactions which could cause the association to increase as the pH is decreased: (1) if there were a pair of interacting carboxyls, as postulated (Timasheff, 1969), pro-

tonation of one would eliminate the unfavorable (repulsive) electrostatic interaction; (2) protonation of a neutral side chain, with the creation of a positive charge, could result in a favorable interaction (with respect to association) with a negatively charged side chain. There are two types of charge interactions which could cause a *decrease* in association with a decrease in pH: (1) the elimination of a favorable (attractive) cationic-anionic interaction by the protonation of the anionic group; (2) the increasing unfavorable long-range electrostatic repulsion between two macroions with a net positive charge. In this analysis, it is assumed that no conformational change occurs as a function of pH. The uptake or release of protons by the protein could conceivably allow certain specific side chains to assume new conformational states, which are removed energetically by more than  $kT$  ( $k$  is the Boltzmann constant) from the "relaxed" macroscopic state. Their fixing in the new conformations by this energy difference could result in the above types of favorable *or* unfavorable electrostatic interactions. Alternately, it could strengthen or weaken interactions between protein molecules by either creating or eliminating hydrogen bonds or properly matching hydrophobic sites.

The most subtle movements of side chains cannot be unambiguously detected either by calorimetry or by optical measurements as a function of temperature, since the enthalpies involved would probably be no greater than the enthalpies of ionization of the proton acceptors. It is known that gross conformational changes can be triggered by the protonation of specific groups (Brandts and Hunt, 1967; Aune and Tanford, 1969; Salahuddin and Tanford, 1970). The question is then: Could such a transition be occurring over the pH range of  $\alpha$ -chymotrypsin dimerization? The denaturation studies of Biltonen and Lumry (1969a,b) show that at 25°, no gross conformational changes take place over pH 2.0. The calorimetric experiments, reported by Shaio and Sturtevant (1970), would also tend to rule out any large enthalpy changes above pH 2.0, where a gross structural change occurs. These studies, therefore, seem to rule out a domination of the pH dependence of dimerization by a major pH-dependent conformational change, such as a denaturation reaction. The question of subtle conformational changes, undetected by optical methods, still remains open.

Assuming that no conformational changes are involved in the dimerization of  $\alpha$ -chymotrypsin, the pH dependence was analyzed in terms of interactions between ionizable groups fixed in space. In the previously reported analysis (Timasheff, 1969), a model involving all of the above mentioned electrostatic considerations was used with total neglect of possible secondary reactions, such as denaturation. The  $pK$ 's of the groups at contact were preassigned and a calculation was made of the pH dependence of the free energy of electrostatic interaction between two touching spherical monomers, without taking into account free-energy contributions from possible shifts in the  $pK$ 's of groups, as these are brought from their equilibrium state in the monomer to their new state in the dimer. In the present study, an alternative approach was selected. In this, the detailed data were utilized to establish the number and kinds of groups involved. A comparison between the two approaches is given later in the text.

If the equilibrium constant observed experimentally is purely a dimerization constant with no secondary reactions, it can be expressed as a function of solvent variables at constant temperature and pressure,  $K = K(a_H, a_W, a_X)$ , where  $a_i$  is the activity of species  $i$ , with H representing protons, W, water, and X, other solute species. Then, any change in the

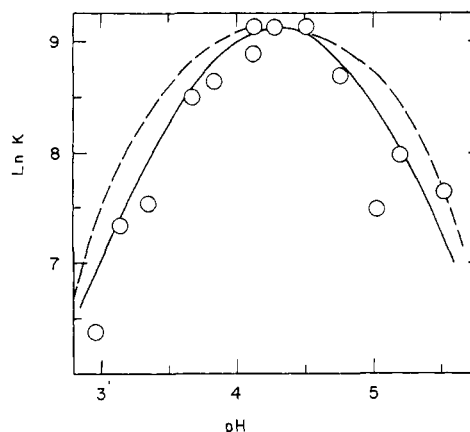


FIGURE 3: Dependence of the natural logarithm of the dimerization equilibrium constant on pH. Conditions: 0.1 M NaCl, 0.01 M acetate buffer, 25°. Circles, experimental points; solid line, theoretical curve calculated using eq 18 and the  $pK_M$  and  $pK_D$  values of Table I; dashed line, theoretical curve calculated using eq 21 and the  $pK_D$  values of Table I, normalized to the maximal experimental value of  $K$ .

dimerization equilibrium constant can be expressed by

$$d \ln K = \left( \frac{\partial \ln K}{\partial \ln a_H} \right)_{a_W, a_X} d \ln a_H + \left( \frac{\partial \ln K}{\partial \ln a_X} \right)_{a_W, a_H} d \ln a_X + \left( \frac{\partial \ln K}{\partial \ln a_W} \right)_{a_X, a_H} d \ln a_W \quad (12)$$

In the range between pH 2.5 and 5.5, it can be assumed that the quantities  $d \ln a_W / d \ln a_H$  and  $d \ln a_X / d \ln a_H$  are negligibly small, so that

$$\frac{d \ln K}{d \ln a_H} = \left( \frac{\partial \ln K}{\partial \ln a_H} \right)_{a_W, a_X} \quad (13)$$

Wyman (1964) has shown that this quantity is

$$\left( \frac{\partial \ln K}{\partial \ln a_i} \right)_{a_j \neq i} = \Delta \bar{\nu}_i \quad (14)$$

where, at constant temperature and pressure,  $K$  is an apparent equilibrium constant for a given reaction,  $a_i$  is the activity of the ligand which perturbs the equilibrium, and  $\Delta \bar{\nu}_i$  is the difference between the numbers of bound ligand molecules to the two states in equilibrium. The partial derivative is taken at a constant activity of all other solvent species,  $j$ , which could perturb the equilibrium. For a dimerization reaction the expression for  $\Delta \bar{\nu}_H$  is given by

$$\Delta \bar{\nu}_H = \bar{\nu}_H^D - 2\bar{\nu}_H^M \quad (15)$$

where the superscripts D and M represent the dimer and monomer, respectively. With a knowledge of  $\Delta \bar{\nu}_H$ , the problem reduces to the writing of a theoretical expression for the relation between  $\bar{\nu}_H^D$  and  $\bar{\nu}_H^M$  which would satisfy the experimental variation of  $\Delta \bar{\nu}_H$ .

Since dimerization reaction neither creates nor destroys the number of ionizable groups on a protein, it may be assumed that the total number of groups is a constant, but that their energy of ionization may be altered. The maximum absolute

value of  $\Delta\bar{v}_H$  deduced from the negative slope in Figure 3 is close to 1.5. The simplest manner of explaining this difference is to assume that only two groups are involved. Thus, in a fashion analogous to the analysis by Aune and Tanford (1969) of the pH dependence of the denaturation of lysozyme, the expression for  $\Delta\bar{v}_H$  is

$$\Delta\bar{v}_H = \frac{2a_H}{a_H + K_{1,D}} + \frac{2a_H}{a_H + K_{2,D}} - 2 \left[ \frac{a_H}{a_H + K_{1,M}} + \frac{a_H}{a_H + K_{2,M}} \right] \quad (16)$$

The coefficient of the bracketed term, 2, is due to the fact that the reaction involves a change in the number of moles of protein kinetic units. The coefficient, 2, of the other terms on the right-hand side of eq 16 expresses the assumption that there is a dyad axis of symmetry in the dimer, such that the environments of groups 1 and 2 in molecule A of the dimer are identical with the environments of groups 1 and 2 in molecule B of the dimer. In the case treated by Aune and Tanford (1969), the ionic strength was above 1, so that long-range electrostatic interactions were negligible. In the present case, however, the ionic strength is below 0.5, and long-range electrostatic interactions are not necessarily unimportant. In fact, in the pH range studied here, taking long-range electrostatic interactions into account might result in a variation by a factor of two in the ionization constants  $K_{1,D}$ ,  $K_{2,D}$ ,  $K_{1,M}$ , and  $K_{2,M}$ , introducing an uncertainty of 0.3 in the pK values. Another consequence of the neglect of the long-range electrostatic interactions is essentially the assumption that no contribution to  $\Delta\bar{v}_H$  is made by other charged groups on the protein which are not directly influenced by or involved in the dimerization reaction. Their proton-binding energies, however, should be affected by the dimerization, with a resulting small contribution to  $\Delta\bar{v}_H$ ; over a narrow pH range, however, this contribution should not vary significantly. A third possible contribution to the pH profile of the dimerization from non-specific electrostatic interactions is the general electrostatic repulsion between the two charged molecules in contact. The change in electrostatic free energy when two charged spherical molecules are brought into contact to form a dimer,  $\Delta F_{\text{dim}}^e$ , is best approximated by the Verwey-Overbeek (1948) equation

$$\Delta F_{\text{dim}}^e = \psi_0^2 \frac{Db^2}{R} e^{-\kappa(R-2b)} \gamma \quad (17)$$

$$\psi_0 = \frac{Z\epsilon}{D} \left( \frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right)$$

where  $D$  is the dielectric constant of the medium,  $b$  is the radius of the monomeric sphere,  $R$  is the center-to-center distance,  $\kappa$  is the Debye-Hückel screening parameter, the reciprocal of the thickness of the double layer,  $\gamma$  is a complicated function of ionic strength and particle separation,  $\psi_0$  is the surface potential on the monomeric molecule,  $Z$  is the net average charge on a monomer,  $\epsilon$  is the electronic charge in esu, and  $a$  is the distance of closest approach between the protein ion and screening small ions in the medium. Using the titration curve of  $\alpha$ -chymotrypsin (Marini and Wunsch, 1963),  $\Delta F_{\text{dim}}^e$  was found to be less than 20% of the experimentally observed free energy of association even at the lowest pH values, i.e., the highest charged state of the protein, studied. Since  $\Delta F_{\text{dim}}^e$  falls rapidly with increasing pH, its contribution could be

neglected in the present studies, without introducing any significant uncertainty.

Since the precision of the data does not allow consideration of all these terms, it was deemed most reasonable to interpret the data in terms of the simplest possible analysis, based on the premise that the overwhelming contribution is made by the direct interaction of the pair of oppositely charged ionizing groups. The pH dependence of the dimerization constant is then given by eq 18, which is obtained by combining eq 16 and 14 and integrating.

$$\ln K = \ln K(\text{pH} = \infty) +$$

$$2 \ln \frac{(1 + a_H/K_{1,D})(1 + a_H/K_{2,D})}{(1 + a_H/K_{1,M})(1 + a_H/K_{2,M})} \quad (18)$$

The data of Figure 3 were then curve fitted to this expression by successive approximation with the aid of the programmable Wang calculator. The results of successive computations were recorded on the teletype and compared with the data until a most reasonable fit was found. It should be noted that deviations by 0.2 of the pK values from those listed in Table I re-

TABLE I: pK's of Groups Involved in Dimerization of  $\alpha$ -Chymotrypsin.

Without Long-Range Electrostatics		With Long-Range Electrostatics	
pK <sub>D</sub>	pK <sub>M</sub>	pK <sub>D</sub>	pK <sub>M</sub>
6.2	5.0	6.2	5.2
2.4	3.6	2.2	4.5

sulted in marked differences between the calculated curve and the experimental points. The resulting values of pK<sub>1,D</sub>, pK<sub>2,D</sub>, pK<sub>1,M</sub>, and pK<sub>2,M</sub>, are given in columns 1 and 2 of Table I. Replacement of  $K_i$  in eq 18 by

$$K_i = K_i^0 e^{2wZ} \quad (19)$$

$$w = \frac{\epsilon^2}{2DkTb} \left( 1 - \frac{\kappa b}{1 + \kappa a} \right)$$

i.e., taking into account the effect on ionization of the long-range electrostatic interactions (Tanford, 1961) due to the overall charge,  $Z$  ( $w$  is the work of charging a group in the presence of other charges on the molecule), resulted in the pK<sub>i</sub><sup>0</sup> values given in columns 3 and 4 of Table I. The charge,  $Z_D$ , for the dimer was taken as twice the charge,  $Z$ , obtained from the titration data of Marini and Wunsch (1963), while the value of  $Z_M$  for the monomer was calculated as

$$Z_M = \frac{Z_D - \Delta\bar{v}_H}{2} \quad (20)$$

This analysis includes the effect of long-range electrostatics only on the two directly interacting groups. Furthermore, this correction can be regarded as no better than an approximation, since eq 14 is not directly integrable when the ionization constants are pH and charge dependent as in eq 19. As seen

from Table I, the effect of long-range electrostatics on the  $pK$  values of the groups in question is not very large, and certainly does not affect their assignment to particular types of residues.

The results in Table I indicate that, during dimerization, two groups shift their  $pK$ 's: one, which ionizes with a  $pK$  of 5.0 in the monomer, shifts to a  $pK$  of about 6.2 in the dimer, the other, with a  $pK$  of 3.6 in the monomer, shifts to about 2.4 in the dimer. This reciprocity in behavior of the two groups implies that a cationic group interacts directly with an anionic group when the dimer is formed. Considering the pH range where the effect is observed, the groups appear to be most likely a terminal or side-chain carboxyl and the imidazole group of a histidine side chain. The good fit of the data by the theoretical curve, calculated with eq 19 and the  $pK$  values of Table I, shows that a single anionic-cationic interaction is sufficient to account for both the necessary free energy and pH dependence of the association. Thus, between pH 2.8 and 5.5, there is no need to invoke the participation of any other specific or nonspecific interactions in the dimerization of  $\alpha$ -chymotrypsin.

The assignment of the groups was further checked by calculating inter- and intramolecular charge-charge distances, in addition to those already provided in the literature (Sigler *et al.*, 1968; Matthews *et al.*, 1967; Blow *et al.*, 1969). This was done from the atomic coordinates of  $\alpha$ -chymotrypsin (Birktoft *et al.*, 1969), with the assumption that the solution dimer corresponds to the asymmetric dimer about dyad axis of symmetry A seen in the crystal structure of this protein. The positions of the charges were considered to be half-way between the oxygen atoms of the carboxyl groups, at the nitrogen atoms of the imidazole groups, at the oxygen atoms of the phenolic groups, at the nitrogen atoms of the amino groups, and half-way between the primary nitrogen atoms of the guanidinium group of arginine.

All the possible intermolecular charge interaction distances, which are less than 10 Å, between the subunits of the asymmetric dimer are shown in the right-hand side of Table II and all the intramolecular charge interactions, less than 5 Å, are shown on the left-hand side of that table. In the absence of a conformational change, only intermolecular charge interactions could affect the pH dependence of association. The possible intermolecular interactions will be examined in turn. One such important interaction could be between aspartic acid 64 and the  $\alpha$ -amino group of residue 149. However, aspartic acid 64 enters already into an intramolecular charge interaction with lysine 36. Therefore, its  $pK$  should be depressed below the pH range of concern in this study. The interaction could contribute to the free energy of formation of the dimer, but it can not contribute to the pH dependence. Likewise, the interaction between the  $\epsilon$ -amino group of lysine 36 and the  $\alpha$ -amino group of residue 149, or that between histidine 57 and the phenolic group of tyrosine 146 would contribute nothing to the pH dependence in the range between pH 2.5 and 5.5.

This leaves then only the interaction between histidine 57 and the  $\alpha$ -carboxyl group of tyrosine 146 as one capable of giving the observed pH dependence. It is known that this interaction is important for dimer formation in the crystal (Sigler *et al.*, 1968; Wright *et al.*, 1968). Therefore, the present interpretation of the solution results indicates that the dimer geometry observed in the crystal about dyad axis A is maintained in solution.

The carboxyl-carboxyl repulsive interaction which was suggested by Egan *et al.* (1957) to account for the pH dependence up to pH 7.5 and used in the model calculation of

TABLE II: Distances between Interacting Groups in  $\alpha$ -Chymotrypsin.

Intramolecular <sup>a</sup> Charge Distances <5 Å (Å)		Intermolecular <sup>b</sup> Charge Distances <10 Å (Å)	
His <sub>57</sub> -Asp <sub>102</sub>	3.5	His <sub>57</sub> - $\alpha$ -COOH <sub>146</sub>	4.7
Lys <sub>36</sub> -Asp <sub>64</sub>	3.1	His <sub>57</sub> -Tyr <sub>146</sub>	2.9
Lys <sub>107</sub> - $\alpha$ -COOH <sub>245</sub>	3.2	Lys <sub>36</sub> - $\alpha$ -NH <sub>3</sub> <sub>149</sub>	4.2
Lys <sub>203</sub> -Asp <sub>128</sub>	4.2	Asp <sub>64</sub> - $\alpha$ -NH <sub>3</sub> <sub>149</sub>	3.7
$\alpha$ -NH <sub>3</sub> <sub>16</sub> -Asp <sub>194</sub>	2.9		

<sup>a</sup> Calculated from coordinates (Birktoft *et al.*, 1969). <sup>b</sup> By means of rotation of coordinates about the dyad axis.

Timasheff (1969) is not found in Table II. Therefore the earlier model (Timasheff, 1969) must be ruled out as not conforming to the structural information. This does not, however, rule out the validity of the method used in calculating the free energy of dimer formation from electrostatic considerations alone (Timasheff, 1969). In fact, the pH dependence of the free energy of association,  $\Delta F^a$ , was calculated by this method for the electrostatic interaction between histidine 57 and the  $\alpha$ -carboxyl of tyrosine 146, using the  $pK$  values of Table I and the electrostatic equation (Timasheff, 1969)

$$\Delta F^a = - \frac{2e^2}{DR_1} e^{-\kappa R_1} \left( \frac{K_{1,D}}{a_H + K_{1,D}} \right) \left( \frac{a_H}{a_H + K_{2,D}} \right) \quad (21)$$

where  $R_1$  is the distance between the groups, equal to 4.7 Å, and the other symbols have their previous meaning. The results of this calculation are shown by the dashed line of Figure 3. As before, the values have been normalized to the maximal value on the experimental pH-dependence curve, since lack of knowledge of the dielectric constant between the charged groups precludes the calculation of absolute values of  $\Delta F^a$ . It is seen that the agreement is reasonable with the experimental data.

It is not possible to make a direct comparison between the two methods of analysis. The electrostatic method uses a relationship where absolute protonic charge is important, while the method used in the present study involves the direct evaluation of the difference in free energy between dimer and monomer in terms of ligand binding. The present method makes it possible to establish the  $pK$ 's of the interacting groups from the pH dependence of the association; the electrostatic method requires prior knowledge of the  $pK$ 's. A combination of the two methods, using in the second the  $pK$ 's deduced by the first, can serve as a good check of the validity of the mechanism. The agreement obtained between the two analyses in the present study strongly supports the conclusion that the observed pH dependence of the association is the result, almost entirely, of electrostatic interactions between the two identical pairs of charged groups. The deviation from the experimental points of the calculated

results, using the electrostatic model, can be ascribed to the fact that in eq 21, only the charge-charge interactions between the sites in the dimer are taken into account. The work of charging these groups in the monomer, which is included in eq 18, is neglected in this calculation.

That tyrosine 146 is important in the dimerization of  $\alpha$ -chymotrypsin has been suggested in a number of studies. Gladner and Neurath (1954) have shown that removal of the group by treatment with carboxypeptidase eliminated the association. The  $\delta$ -chymotrypsin molecule, in which tyrosine 146 is in a peptide linkage, does not dimerize, as has been shown by Neet and Brydon (1970) in a sedimentation velocity study, and by the method of sedimentation equilibrium by Horbett and Teller (1970) and in this laboratory. These observations do not prove the role of the  $\alpha$ -carboxyl group, however, since the conformation of the phenolic ring could also be very important in providing the correct environment for the dimerization.

Although  $\gamma$ -chymotrypsin is chemically identical with  $\alpha$ -chymotrypsin, and therefore contains a C-terminal tyrosine 146, there is reason to believe that the conformations of some of the side chains differ in the two forms (Corey *et al.*, 1965; Wright *et al.*, 1968; Matthews *et al.*, 1969). Because the crystal structures differ, it is clear that the surface charge interactions are likely to be different. The question as to whether  $\gamma$ -chymotrypsin dimerizes and, if it does, whether it goes through the same mode as  $\alpha$ -chymotrypsin has been answered only partially. Schwert (1949) concluded from a sedimentation velocity study that  $\gamma$ -chymotrypsin had a smaller tendency to dimerize than  $\alpha$ -chymotrypsin. This result was confirmed in this laboratory by the method of sedimentation equilibrium. Preliminary results have shown a weak association. This may mean that the sample of enzymatically fully active  $\gamma$ -chymotrypsin used was either a mixture of nonassociating  $\gamma$ -chymotrypsin and associating  $\alpha$ -chymotrypsin or that, in fact,  $\gamma$ -chymotrypsin dimerizes to a much smaller degree. The latter would imply that the space coordinates of tyrosine 146 vary slightly between the two proteins (histidine 57 is probably in the same position in  $\gamma$ - and  $\alpha$ -chymotrypsins since their activities are essentially equivalent). The discussion of Matthews *et al.* (1968) concerning the conformation of tyrosine 146 in  $\gamma$ - and  $\alpha$ -chymotrypsins lends support to this notion, as do results on the relative accessibilities to chemical modification of the tyrosines of the two enzymes (Gorbunoff, 1971).

The identification of histidine 57 as being involved in the dimerization reaction implies that the active site is in the contact region, a question that has been the subject of much earlier discussion (Schwert and Kaufman, 1951; Smith and Brown, 1952; Neurath and Dreyer, 1955; Egan *et al.*, 1957; Kézdy and Bender, 1965; Morimoto and Kegeles, 1967). The activity, however, is only partially affected, since the crystal is enzymatically active (Kallos, 1964) and small acyl derivatives do dimerize (Neet and Brydon, 1970; Horbett and Teller, 1970).

## Summary

The pH dependence of the dimerization of  $\alpha$ -chymotrypsin has been determined by the method of sedimentation equilibrium. The results are consistent with the proposal that the imidazole group of histidine 57 interacts directly with the  $\alpha$ -carboxyl group of tyrosine 146 to provide part of the free energy of association and to account completely for the pH dependence in the range of pH 2.5–5.5.

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## References

- Aune, K. C., Goldsmith, L. C., and Timasheff, S. N. (1971), *Biochemistry* 10, 1617.
- Aune, K. C., and Tanford, C. (1969), *Biochemistry* 8, 4579.
- Bethune, J. L., and Kegeles, G. (1961), *J. Phys. Chem.* 65, 1761.
- Biltonen, R. L., and Lumry, R. (1969a), *J. Amer. Chem. Soc.* 91, 4251.
- Biltonen, R. L., and Lumry, R. (1969b), *J. Amer. Chem. Soc.* 91, 4256.
- Birktoft, J. J., Matthews, B. W., and Blow, D. M. (1969), *Biochem. Biophys. Res. Commun.* 36, 131.
- Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London)* 221, 337.
- Brandts, J., and Hunt, L. (1967), *J. Amer. Chem. Soc.* 89, 4826.
- Brown, J. R., and Hartley, B. S. (1966a), *Biochem. J.* 101, 214.
- Brown, J. R., and Hartley, B. S. (1966b), *Biochem. J.* 101, 229.
- Corey, R. B., Battfay, O., Brueckner, D. A., and Mark, F. G. (1965), *Biochim. Biophys. Acta* 94, 535.
- Egan, R., Michel, H. O., Schlueter, R., and Jandorf, B. J. (1957), *Arch. Biochem. Biophys.* 66, 366.
- Gilbert, G. A. (1955), *Discuss. Faraday Soc.* 20, 32.
- Gladner, J. A., and Neurath, H. (1954), *J. Biol. Chem.* 206, 911.
- Gorbunoff, M. J. (1971), *Biochemistry* 10, 250.
- Hoagland, V. D., and Teller, D. C. (1969), *Biochemistry* 8, 594.
- Horbett, T. A., and Teller, D. C. (1970), Joint Conference of the Chemical Institute of Canada and the American Chemical Society, Toronto, Canada, May, COLL 14.
- Kallos, J. (1964), *Biochim. Biophys. Acta* 89, 364.
- Kézdy, F. J., and Bender, M. L. (1965), *Biochemistry* 4, 104.
- Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966), *Biochemistry* 5, 365.
- Marini, M. A., and Wunsch, C. (1963), *Biochemistry* 2, 1454.
- Massey, V., Harrington, W. F., and Hartley, B. S. (1955), *Discuss. Faraday Soc.* 20, 24.
- Matthews, B. W., Cohen, G. H., Silverton, E. W., Braxton, H., and Davies, D. R. (1968), *J. Mol. Biol.* 36, 179.
- Matthews, B. W., Sigler, P. B., Genderson, R., and Blow, D. M. (1967), *Nature (London)* 214, 652.
- Monod, J., Changeux, J.-P., and Jacob, F. (1963), *J. Mol. Biol.* 6, 306.
- Morimoto, K., and Kegeles, G. (1967), *Biochemistry* 6, 3007.
- Neet, K. E., and Brydon, S. E. (1970), *Arch. Biochem. Biophys.* 136, 223.
- Neurath, H., and Dreyer, W. J. (1955), *Discuss. Faraday Soc.* 20, 32.
- Rao, M. S. N., and Kegeles, G. (1958), *J. Amer. Chem. Soc.* 80, 5724.
- Roark, D. E., and Yphantis, D. A. (1969), *Ann. N. Y. Acad. Sci.* 164, 245.
- Salahuddin, A., and Tanford, C. (1969), *Biochemistry* 8, 4579.
- Sarfare, P. S., Kegeles, G., and Kwan-Rhee, S. Ja (1966), *Biochemistry* 5, 1389.
- Schwert, G. W. (1949), *J. Biol. Chem.* 179, 655.



- Schwert, G. W., and Kaufman, S. (1951), *J. Biol. Chem.* **190**, 807.
- Shiao, D. D. F., and Sturtevant, J. M. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **29**, 505.
- Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol.* **35**, 143.
- Smith, E. L., and Brown, D. M. (1952), *J. Biol. Chem.* **195**, 525.
- Steiner, R. F. (1954), *Arch. Biochem. Biophys.* **53**, 457.
- Tanford, C. (1961), *Physical Chemistry of Macromolecules*, New York, N. Y., Wiley, Chapter 7.
- Teller, D. C., Horbett, T. A., Richards, G. E., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci.* **164**, 66.
- Timasheff, S. N. (1969), *Arch. Biochem. Biophys.* **132**, 165.
- Tinoco, I., Jr. (1957), *Arch. Biochem. Biophys.* **68**, 367.
- Verwey, E. J., and Overbeek, J. Th. G. (1958), *Theory of the Stability of Lyophobic Colloids*, Amsterdam, Elsevier.
- Wright, H. T., Kraut, J., and Wilcox, P. E. (1968), *J. Mol. Biol.* **37**, 363.
- Wyman, J. (1964), *Advan. Protein Chem.* **19**, 224.
- Yphantis, D. A. (1964), *Biochemistry* **3**, 297.

## Dimerization of $\alpha$ -Chymotrypsin. II. Ionic Strength and Temperature Dependence\*

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**ABSTRACT:** The acid dimerization equilibrium constant of  $\alpha$ -chymotrypsin was determined by the method of sedimentation equilibrium as a function of ionic strength and temperature. It was found that this reaction is accompanied by the apparent preferential binding of salt, a negative change in heat capacity, and a positive entropy at low temperature. Change of medium from H<sub>2</sub>O to D<sub>2</sub>O enhanced the dimeriza-

tion. Perchlorate ions were found to strengthen dimer formation, while sulfate weakened it. Analysis of these thermodynamic observations led to the conclusions that water leaves the domain of the intermolecular contact region when the dimer forms and that the released water comes not only from hydrophobic sites, but also from the hydration shells of charged groups in the contact region.

In the preceding study (Aune and Timasheff, 1971) an analysis of the pH dependence of the dimerization of  $\alpha$ -chymotrypsin below pH 5.5 revealed that it could be completely accounted for in terms of two specific interacting charged groups in the dimer. These groups were identified as being most likely the imidazole group of histidine 57 and the  $\alpha$ -carboxyl group of tyrosine 146.

In earlier studies, Steiner (1954) and Egan *et al.* (1957) had found that an increase in ionic strength enhanced the association of  $\alpha$ -chymotrypsin in the acid pH region. Steiner (1954) interpreted this effect in terms of the diminution of unfavorable electrostatic repulsion. The temperature dependence of turbidity at a single protein concentration further led Steiner (1954) to conclude that the enthalpy of dimerization was very small or zero. In the present study, it has been found that the nonspecific ionic strength effect might be

of minor importance, the predominant effect of salt being a change in preferential solvation in the contact region of the dimer. This change is reflected in a temperature dependence of the association. The significance of these observations to the mechanism of  $\alpha$ -chymotrypsin dimerization is discussed in this paper.

### Materials and Methods

The deuterium oxide used in this study was Bio-Rad<sup>1</sup> Lot No. 7056. The density and viscosity of deuterium oxide used were those compiled by Kirshenbaum (1951). The sedimentation coefficient of  $\alpha$ -chymotrypsin was measured in the conventional manner on a Spinco Model E analytical ultracentrifuge at 56,000 rpm. The schlieren patterns as a function of time were recorded on Kodak metallographic plates and peak positions were measured on a Nikon 6C microcomparator. The least-squares slope of the natural logarithm of the peak radial position *vs.* time was computed and the results were corrected according to Svedberg and Pederson (1940) to the conditions of hydrogen oxide at 20° on a Wang 370 electronic programmable calculator. The partial specific volume of  $\alpha$ -chymotrypsin in deuterium oxide was assumed to be lowered by a factor of  $1/1.0155$  relative to that in hydrogen oxide, because of hydrogen exchange without volume change (Hvidt and Nielson, 1966; Edelstein and

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