3104

On the Mechanism of α-Chymotrypsin Dimerization

SERGE N. TIMASHEFF

Pioneering Laboratory for Physical Biochemistry, United States Department of Agriculture; and Graduate Department of Biochemistry,[1] *Brandeis University, Waltham, Massachusetts 02154*

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The dimerization of α-chymotrypsin between pH 2.5 and 8 has been examined in terms of electrostatic interactions. In appears that the association involves the attraction between a pair of groups with pK values of 3.3 and 8.5 and the repulsion between a pair of carboxyls with pK 5.0.

The ability of α-chymotrypsin to undergo self-association reactions has been known for a long time (1–13). At acid pH values and an ionic strength of 0.1, a dimer seems to be the prevalent aggregated species (3, 8). In an ultracentrifugal study, Egan *et al.* (8) have shown that the pH dependence of this association is of a bell-shaped type; above pH 2.3, the sedimentation coefficient rises sharply, passes through a maximum at pH 3.5–4.5, and then decreases slowly until pH 8.6. Such a pH dependence suggests the participation of ionizable groups in this reaction (3, 8, 13). The results of recent crystallographic studies (14–18) have made it possible to test this hypothesis. Blow *et al.* (14) have suggested that the presence of dyad axis of symmetry A between α-chymotrypsin molecules in crystals is related to the ability of these molecules to dimerize in solution. Furthermore, knowledge of interactions about the dyad axis between specific groups on the two proteins (16) renders possible calculations of the electrostatic free energies of the dimerization as a function of pH.

The experimental free energies of α-chymotrypsin dimerization were calculated from the sedimentation velocity data of Egan *et al.* (8), using the Gilbert theory of sedimentation velocity of aggregating systems in rapidly reversed equilibrium (19, 20). Gilbert has shown that, for a system in rapid equilibrium, the equilibrium dissociation constant, K_G (in grams/liter), is related to the protein concentration, C (in grams/liter), the degree of polymerization, n, and the sedimentation velocity parameter, δ, by the equation:[2]

$$K_G = \frac{n(1-\delta)}{\delta} \frac{C^{n-1}}{\left(1 + \frac{1}{n}\frac{\delta}{1-\delta}\right)^{n-1}} \quad (1)$$

The parameter δ is the sedimentation rate of matter at any given position in the boundary, relative to a stationary monomer. Thus, if for the reaction $n\mathrm{M} \rightleftharpoons \mathrm{A}$, s_M, s_A, and s_i are the sedimentation coefficients of monomer, aggregate and material at position i in the boundary, then

$$\delta = \frac{s_i - s_M}{s_A - s_M} \quad (2)$$

Furthermore, since in such associating systems the addition of macromolecular material (increase in concentration) develops the

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[2] This neglects diffusion and the hydrodynamic concentration-dependent retardation; these can be accounted for in semi-empirical manner (21–23).

boundary on the rapid side only, the slow side remaining unchanged, a single construction gives the entire concentration dependence of the system.

Using Eqs. (1) and (2), and taking the sedimentation coefficients of monomer and dimer as 2.6S and 4.0S, respectively, values of K_G for the dimerization of α-chymotrypsin were calculated as a function of pH from the concentration dependence of s_{20} reported by Egan *et al.* (8). From these, the standard free energy of association, $\Delta F°$, in Kcal/mole, was calculated from the relation

$$\Delta F° = -2.303\ RT \log \frac{M_M}{2K_G} \qquad (3)$$

where R is the gas constant, T is the thermodynamic temperature and M_M is the monomer molecular weight, taken as 22,500. The free energies of dimerization calculated in this manner are listed in Table I as a function of pH; they are found to be in reasonable agreement with values determined by Steiner (3) in light scattering studies.

The pattern of interaction used in the present calculations is represented schematically in Fig. 1. Each monomer is assumed to be a sphere of radius b with a specific site of dimerization. Each site contains an anionic group A and a cationic group B which interact in pairs A_1–B_2 and

TABLE I

CALCULATED PARAMETERS OF α-CHYMOTRYPSIN DIMERIZATION

pH	K_G (grams/liter)	$\Delta F°$ (Kcal/mole) Calculated from: Egan *et al.* (8)	Steiner (3)
2.7	37.6	−3.4	
3.0	14.3	−3.9	
3.6	4.1	−4.7	
3.7			−5.0
4.1	2.8	−4.9	
4.4			−4.6
4.6	4.1	−4.7	
5.2			−4.3
5.6	14.3	−3.9	
6.2			−4.2
7.2	37.6	−3.4	

Dimerization of α-Chymotrypsin

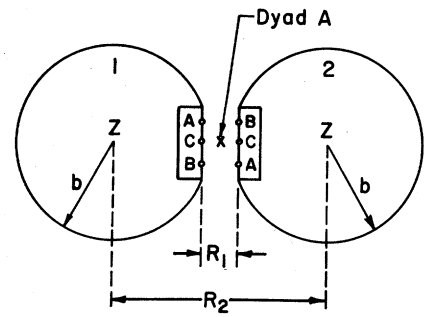

FIG. 1. Schematic model of α-chymotrypsin dimerization. The dyad axis of symmetry A is perpendicular to the plane of the paper.

B_1–A_2 about dyad axis A, as well as an anionic group C. The groups C are brought into mutual proximity by the interaction and repel each other. Furthermore, each spherical monomer carries a net average charge Z evenly distributed over the surface, in accordance with the Linderstrøm-Lang model of charge distribution on protein molecules (24). The center-to-center distance between the two spheres is R_2, while the distance between the two sites is R_1. Using screened Coulombic potentials for interactions between specific groups and the Verwey-Overbeek potential for the general electrostatic repulsion between the two charged spheres (25), the potential of interaction between the two spheres, $W(R)$, is given by (26)

$$W(R) = \frac{2Z_A Z_B \epsilon^2}{DR_1} e^{-2\kappa R_1} + \frac{Z_C^2 \epsilon^2}{DR_1} e^{-2\kappa R_1} + \psi_0^2 \frac{Db^2}{R_2} e^{-\kappa(R_2-2b)} \gamma, \qquad (4)$$

where Z_A is the time average valence of group A at the given pH, Z_B that of group B, Z_C that of C, ϵ is the electronic charge, D is the dielectric constant, κ is the Debye-Hückel screening parameter, γ is a complicated function of ionic strength and particle separation (25), and ψ_0 is the surface poten-

tial on the molecule:

$$\psi_0 = \frac{Ze}{D}\left(\frac{1}{b} + \frac{\kappa}{1 + \kappa a}\right), \qquad (5)$$

where a is the distance of closest approach between the protein ion and screening small ions in the medium. Since in the pH range of interest, the specific groups involved are undergoing ionization, a more convenient form of Eq. (4) is:

$$W(R) = \frac{\epsilon^2}{DR_1} e^{-\kappa R_1}\left[\frac{K_C{}^2 e^{4wZ}}{([H^+] + K_C e^{2wZ})^2} - 2\left(\frac{K_A e^{2wZ}}{[H^+] + K_A e^{2wZ}}\right)\left(\frac{[H^+]}{[H^+] + K_B e^{2wZ}}\right)\right] + \psi_0{}^2 \frac{Db^2}{R_2} e^{-\kappa(R_2 - 2b)}\gamma, \qquad (6)$$

where $[H^+]$ is the hydrogen ion concentration, K_A, K_B, and K_C are the ionization constants of groups A, B, and C and w is the work function of charging a group in the presence of other charges on the molecule:

$$w = \frac{\epsilon^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \qquad (7)$$

where k is the Boltzmann constant.

Calculations were carried out using Eq. (6), the titration curve of α-chymotrypsin (27) and various values of K_A, K_B and K_C, as well as of the distance R_1; R_2 was set equal to $2b$, i.e., to twice the radius of the sphere, taken as 20 Å; κ was taken as 1.04×10^7 cm^{-1}, i.e., for an ionic strength of 0.1. It was found that the best fit of the experimental results was given with $K_A = 5 \times 10^{-4}$; $K_B = 3 \times 10^{-9}$ and $K_C = 1 \times 10^{-5}$ and $R_1 = 2$ Å. The results, presented as $\Delta F^e/\Delta F^e_{max}$, are compared with the experimental values in Fig. 2, and reasonable agreement is obtained for the pH dependence. The free energies are expressed as a ratio to the maximal value, since the absolute values may be changed by varying the values of the dielectric constant or the distance R_1 in Eq. (6).

The ionization constants of the groups involved in the interaction correspond to

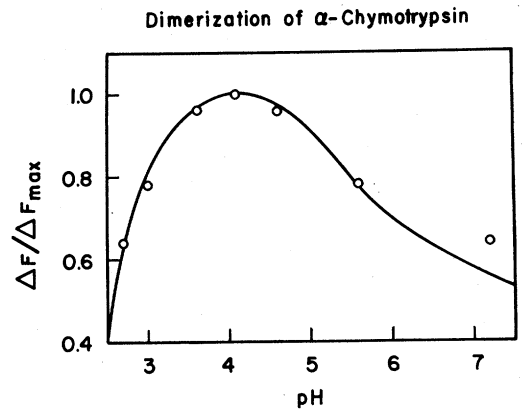

FIG. 2. Comparison of the free energies of α-chymotrypsin dimerization, calculated from the data of Egan *et al.* (8), with those calculated with Eq. (6) according to the model of Fig. 1.

pK values of 3.3, 8.53, and 5.0 for groups A, B, and C, respectively. Examining the results of X-ray diffraction studies, the attractive pairs A–B should correspond most probably to the interaction between the β-COO$^-$ of aspartate 64 and the α-NH$_3^+$ of alanine 149 (16). A pK of 3.3 is within limits for an aspartic acid residue (28) included in the complex charge environment of the protein molecule, possibly in the vicinity of positively charged groups, such as histidine 57 and the α-amino of alanine 149. Alternately, the anionic group could be the α-COO$^-$ of tyrosine 146; this, however, would require a small conformational change, which is not excluded (18). That tyrosine 146 may be involved in the dimerization reaction is suggested by the fact that its removal with carboxypeptidase yields a product incapable of forming dimers (7). A pK of 8.5 is reasonable for a terminal amino group (29). The third, C–C, pair could correspond to side chain carboxyls located in the immediate vicinity of the interacting site; there is, however, no information in the literature bearing on this point. The distance R_1 of 2 Å is reasonable for closest approach between a pair of interacting charges (30). Variation of this parameter, however, would affect only the abso-

lute values of ΔF° without affecting the ratio, $\Delta F^e/\Delta F^e_{\max}$.

The present calculations indicate that the pH dependence of α-chymotrypsin dimerization can be accounted for in terms of electrostatic interactions, -specific attraction and repulsion between the three groups, A, B, and C and general nonspecific repulsion. That other forces are involved in the binding energy is quite certain (31). Thus, contacts between hydrophobic residues, such as between the pair of methionines 192 (16), must make a contribution. Similarly, hydrogen bonding may be involved, possibly between the phenolic group of tyrosine 146 and histidine 57 (16). These interactions, however, may be expected to have little effect on the pH dependence of the association. Since constant contributions over the pH range to the free energy of dimerization can be expected from these types of interactions, the electrostatic contribution to the free energy of association, ΔF^e, at any pH is:

$$\Delta F^e = \Delta F^\circ - \Delta F^{ne}, \qquad (8)$$

where ΔF° is the experimentally observed free energy and ΔF^{ne} is the contribution from nonelectrostatic effects. The ratio $\Delta F^\circ/\Delta F^\circ_{\max}$, calculated as a function of pH, gives then, maximal possible values for $\Delta F^e/\Delta F^e_{\max}$, with $\Delta F^{ne} = 0$. In actuality the calculated ratios should be compared with experimental quantities smaller than those given in Fig. 2, i.e., with $(\Delta F^\circ - \Delta F^{ne})/(\Delta F^\circ_{\max} - \Delta F^{ne})$. Such a correction would raise the value of pK_A and lower that of pK_B, i.e., it would bring these values in closer agreement with the dissociation constants normally assigned to aspartate β-carboxyl and α-amino groups.

REFERENCES

1. Schwert, G. W., *J. Biol. Chem.* **179,** 655 (1949).
2. Schwert, G. W. and Kaufman, S., *J. Biol. Chem.* **190,** 807 (1951).
3. Steiner, R. F., *Arch. Biochem. Biophys.* **53,** 457 (1954).
4. Tinoco, I., *Arch. Biochem. Biophys.* **68,** 367 (1957).
5. Massey, V., Harrington, W. F., and Hartley, B. S., *Discussions Faraday Soc.* **20,** 24 (1955).
6. Neurath, H. and Dreyer, W. J., *Discussions Faraday Soc.* **20,** 32 (1955).
7. Gladner, J. A. and Neurath, H., *J. Biol. Chem.* **206,** 911 (1954).
8. Egan, R., Michel, H. O., Schlueter, R., and Jandorf, B. J., *Arch. Biochem. Biophys.* **66,** 366 (1957).
9. Rao, M. S. N. and Kegeles, G., *J. Am. Chem. Soc.* **80,** 5724 (1958).
10. Bethune, J. L. and Kegeles, G., *J. Phys. Chem.* **65,** 1761 (1961).
11. Kézdy, F. J. and Bender, M. L., *Biochemistry* **4,** 104 (1965).
12. Sarfare, P. S., Kegeles, G., and Kwon-Rhee, S. J., *Bicochemistry* **5,** 1389 (1966).
13. Smith, E. L. and Brown, D. M., *J. Biol. Chem.* **195,** 525 (1952).
14. Blow, D. M., Rossman, M. G., and Jeffrey, B. A., *J. Mol. Biol.* **8,** 65 (1964).
15. Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M., *Nature* **214,** 652 (1967).
16. Sigler, P. B., Blow, D. M., Matthews, B. W. and Henderson, R., *J. Mol. Biol.*, **35,** 143 (1968).
17. Matthews, B. W., Cohen, G. H., Silverton, E. W., Braxton, H., and Davies, D. R., *J. Mol. Biol.* **36,** 179 (1968).
18. Wright, H. T., Kraut, J., and Wilcox, P. E., *J. Mol. Biol.* **37,** 363 (1968).
19. Gilbert, G. A., *Discussions Faraday Soc.* **20,** 68 (1955).
20. Gilbert, G. A., *Proc. Roy. Soc. A* **250,** 377 (1959).
21. Gilbert, G. A., *Proc. Roy. Soc.* (*London*) **A276,** 354 (1963).
22. Townend, R., Winterbottom, R. J., and Timasheff, S. N., *J. Am. Chem. Soc.* **82,** 3161 (1960).
23. Timasheff, S. N. and Townend, R., *J. Am. Chem. Soc.* **83,** 464 (1961).
24. Linderstrøm-Lang, K. V., Compt. Rend. Trav. Lab. Carlsberg, **15,** 1 (1924).
25. Verwey, E. J. and Overbeek, J. Th. G., "Theory of the Stability of Lyophobic Colloids," Elsevier, Amsterdam, 1948.
26. Timasheff, S. N., Polyelectrolyte properties of globular proteins, *In* "Physical Chemistry of Polyelectrolytes" (A. Veis, ed.), Dekker, N. Y., 1969, in press.

27. Marini, M. A. and Wunsch, C., *Biochemistry* **2,** 1454 (1963).
28. Nozaki, Y. and Tanford, C., *J. Biol. Chem.* **242,** 4731 (1967).
29. Tanford, C., *Adv. Protein Chem.* **17,** 69 (1962).
30. Tanford, C., *J. Am. Chem. Soc.* **79,** 5348 (1957).
31. Timasheff, S. N., *In* "Proteins and their reactions" (H. W. Schultz and A. F. Anglemeier, eds.) p. 179. Avi Publishing Co., Wesport, Conn, 1964.