Characterization of the Self-Association of a Soybean Proteinase Inhibitor by Membrane Osmometry*

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ABSTRACT: The self-association of a soybean trypsin and chymotrypsin inhibitor has been characterized by membrane osmometry. The association is found to be of the monomer $\rightleftharpoons$ dimer type with little or no higher forms present. The extent of association increases with increased ionic strength and with decreased monomer charge. The ionic strength effect and the pH effect (below pH 6) are both found to be consistent with the theoretical electrostatic free-energy changes.

Recent results from sedimentation equilibrium studies in this laboratory (Millar *et al.*, 1969) have shown the Bowman–Birk[1] soybean inhibitor of trypsin and chymotrypsin to be a reversibly associating system. However, the molecular weights reported were in conflict with that found by Birk *et al.* (1963). Because of this ambiguity concerning molecular weight, and because of the similarities which have been shown to exist between various soybean and lima bean inhibitors (Jones *et al.*, 1963; Haynes and Feeney, 1967; Frattali, 1969), it would be beneficial to investigate the dependence of the association process on pH and ionic strength. Such a characterization would also be of interest in terms of providing some insight as to the forces responsible for the self-interaction of the protein.

This paper describes such a study; utilizing a commercial, high-speed membrane osmometer to characterize the association. The use of such instruments as investigative tools in protein structural research was first explored by Banerjee and Lauffer (1966), and utilized recently by Jaenicke *et al.* (1968) and by Castellino and Barker (1968). Osmotic pressure was a suitable tool for use in this study because the molecular weight range involved was low enough to give large observable osmotic pressures, and therefore greater reliability, in the concentration range in which dissociation occurred.

Materials

Commercial Bowman–Birk inhibitor was obtained from Miles Laboratories, Inc., Elkhart, Ind. The inhibitor was freed of several minor contaminants by preparative electrophoretic purification on polyacrylamide gel, using a procedure previously described (Frattali and Steiner, 1969). Combined fractions were exhaustively dialyzed against glass-distilled water, lyophilized, and stored at 5°.

Buffers were prepared with analytical grade reagents and glass-distilled water. The base used in the titration was 1 N carbonate-free sodium hydroxide, standardized against potassium acid phthalate. The acid used was 1 N hydrochloric, standardized against the sodium hydroxide.

Methods

Osmometer. Osmotic pressure measurements were made with a Hewlett–Packard Model 503 high-speed membrane osmometer equipped with a strip chart recorder. This is a self-balancing instrument which utilizes a photoelectric detector, coupled with a servomechanism, to measure osmotic flow. Some skill is necessary for proper assembly of the instrument and the paper by Paglini (1968) was found to be helpful in this regard. Measurements were made at 25 or 5 ± 0.2°.

Membranes. A major problem in studying a system with a monomer molecular weight below 10,000 by osmometry is in finding a semipermeable membrane whose pore size is small enough to retain the protein, but yet will permit adequate solvent transport. The membranes used in this study were Schleicher & Schuell (Keene, N. H.) type B-20, cellulose acetate membranes, with an average pore size reported by the manufacturer to be 5 mμ. Various molecular weight retention values are to be found for this membrane, ranging from a low of 7,000 (Armstrong, 1968) to a high of 50,000 (Schleicher & Schuell, Bulletin No. 90). We have observed large variation in retentive ability from membrane to membrane, some being eminently suitable for our purpose; others resulting in osmotic pressure decreases (signifying membrane permeation) of greater than 30%/hr.

The zero flow pressures were determined as a function of time, allowing the course of protein permeation to be followed. The error due to protein permeation was minimized by extrapolation of osmotic pressures to zero time (the time of filling of the osmometer). Only data from those runs in which the decrease in osmotic pressure was no greater than 8%/hr have been utilized in the calculations. In order to reduce somewhat the haphazard nature of proper membrane selection, a double thickness of membranes was used in some runs. Data from these runs were reproducible with those using a single membrane.

Preparation of Solutions. Lyophilized inhibitor was dissolved in the selected buffer and dialyzed against the buffer for at least 5 hr. The buffers employed were: (pH 7) 0.01 M potas-

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[1] Inhibitor "AA" of Birk *et al.* (1963).

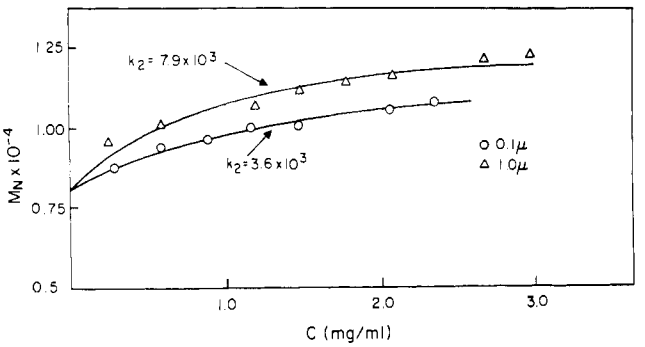

FIGURE 1: Effect of ionic strength on the association of the Bowman–Birk Inhibitor.

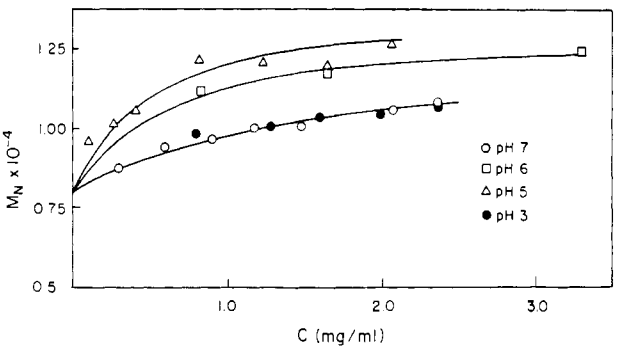

FIGURE 2: Effect of pH on the association of the Bowman–Birk Inhibitor.

sium phosphate, (pH 6) 0.01 M potassium phosphate, (pH 5) 0.01 M acetate, and (pH 3) 0.01 M glycine. In addition all buffers contained potassium chloride (0.1 or 1.0 M) and 0.001 M EDTA. No detergents were employed either in the protein solutions or in the solvent in the capillary.

The concentration of the dialyzed inhibitor was determined by measuring the optical density in a Gilford Model 2000 spectrophotometer. A value of 4.4 was used as the extinction coefficient at 280 mμ for a 1% solution in a 1.0-cm cell (Frattali, 1969). A series of dilutions was prepared from the stock and these were run in the osmometer in order of increasing concentration. The base-line value (buffer *vs.* buffer) was repeated after each dilution and was found to decrease slightly, but reproducibly, each time. Generally, osmotic pressure runs were made within 24 hr of dialysis.

The number-average molecular weight of the inhibitor was determined at each concentration from eq 1, assuming ideality.

$$M_n = \frac{RTc}{\pi} \tag{1}$$

Osmotic pressure, π, is obtained in centimeters of buffer, and the gas constant, R, must be converted into pressure units of centimeters of buffer. The buffer densities employed were those for 0.1 or 1.0 M potassium chloride.

Titration. The titration of the inhibitor was carried out on a Corning Model 12 research pH meter equipped with a Corning combination glass electrode. Protein (31.8 mg) was taken up in 10 ml of 0.1 M KCl and titrated in a water-jacketed beaker equipped with a magnetic stirrer. The temperature inside the jacket was maintained at 25 ± 0.1° by circulation of water from a thermostated bath. The pH meter was calibrated with phosphate buffer before and after the titration, the variation being less than 0.01 pH unit. A stream of nitrogen was passed through soda lime and bubbled through water, and then bubbled through the solution. The beaker was covered with rubber dental dam, holes being punched for the electrode, buret, and nitrogen stream; this in effect gave a sealed titration vessel. Acid and base solutions were added from a 1.0-cc Manostat Corp. microburet. The titration of a solvent blank was treated in a similar manner and the titration curve of the protein was obtained by subtraction of the solvent curve from that of the protein solution. The titration curve below pH 3.5 was corrected for the activity coefficient of H^+ in water at 25° (Lange's Handbook of Chemistry, 1967).

Evaluation of Equilibrium Constants. The equilibrium constants for dimer formation were calculated by the method of Steiner (1954) for consecutive association constants, assuming ideal behavior. The method involves computing the mole fraction of monomer units from the observed concentration dependence of number-average molecular weight. Steiner demonstrated that

$$\ln Z_1 = \int_0^m \frac{(\alpha_n)^{-1} - 1}{m} dm \tag{2}$$

where Z_1 = mole fraction of monomer units, m = total molar concentration of protein, α_n = number-average degree of association = M_n/M_1,M_n, = number-average molecular weight, and M_1 = monomer molecular weight. The total molar concentration of protein is

$$m = C/M_n \tag{3}$$

where C is the total weight concentration, and the molar concentration of monomer is

$$m_1 = Z_1 m \tag{4}$$

The consecutive association constants are then found from the equation

$$m = m_1 + k_2 m_1^2 + k_2 k_3 m_1^3 + \ldots . \tag{5}$$

Plots of m_1 *vs.* m/m_1 were always linear for this case.

Calculations were carried out on a General Electric Mark I time-sharing computer. The integration of eq 2 was accomplished by employing the trapezoidal rule.

Results

Figures 1 and 2, along with Table I summarize the data obtained concerning the association process under various conditions of pH and ionic strength. For this system the monomer molecular weight is estimated to be 8000, based on the sedimentation equilibrium studies (Millar *et al.*, 1969) and the fact that the minimum molecular weight calculated from the amino acid analysis is 7975 (Frattali, 1969). As can be seen from the figures, the data extrapolate reasonably well to the

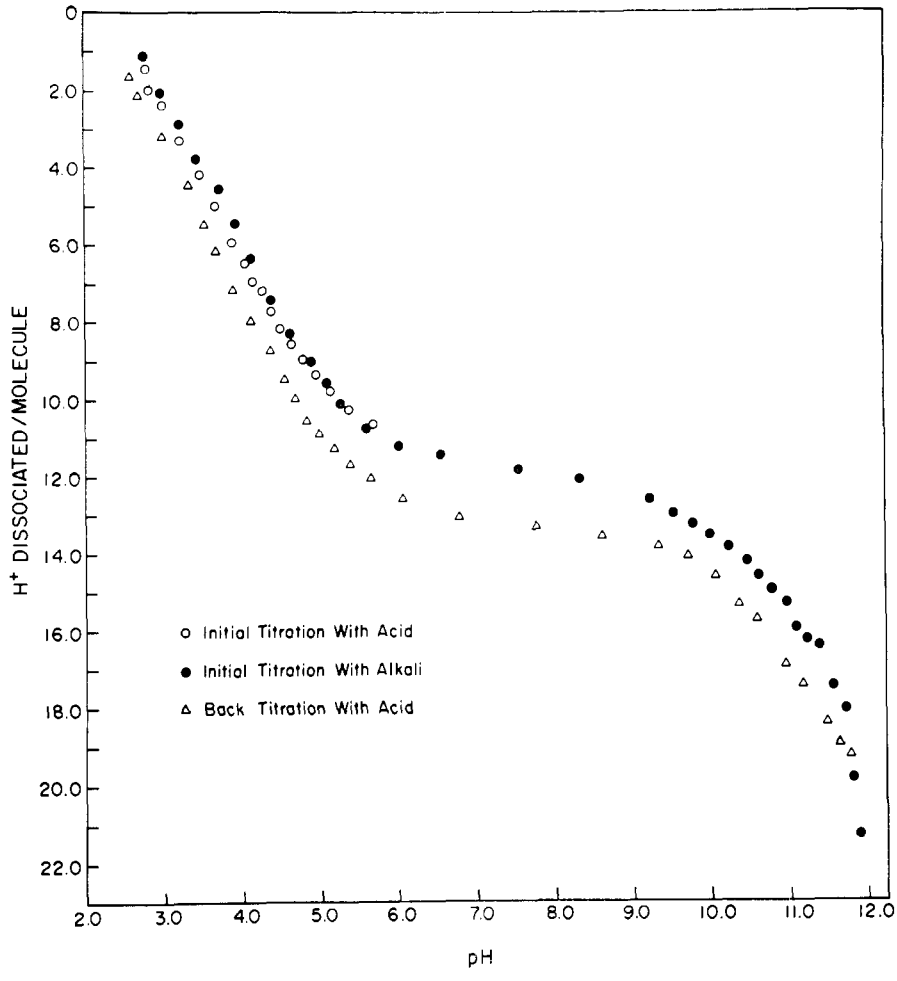

FIGURE 3: Titration curve of the Bowman–Birk inhibitor in 0.1 M KCl at 25°.

presumed monomer molecular weight of 8000. The calculation of equilibrium constants was based on the best line of sight curve through the experimental points.

The points shown in Figures 1 and 2 are experimental ones for a single run under the stated solvent conditions. The curves drawn though the points are based on the calculated equilibrium constants for a monomer $\rightleftharpoons$ dimer equilibrium.

The equilibrium constants (moles per liter) for dimer formation are shown in Table I. The values presented represent the average of at least two runs in each solvent. The number of replicate determinations did not allow for meaningful statistical analyses of confidence limits; however the maximum deviation for any of the solvent systems indicated a possible error of 30%. The relative error in the free energies is, of course, much smaller than that for the corresponding equilibrium constants.

The equilibrium constants at two different temperatures at pH 5 would indicate a zero enthalpy of association. This should probably be interpreted as an apparent zero enthalpy with probable confidence limits of 1.5 kcal. The entropy of association based on an apparent zero enthalpy is approximately 19 entropy units.

Figure 3 is the titration curve of the inhibitor. As can be seen from the figure the titration is reversible below neutrality, but after titration to high pH the titration curves are no longer superimposable, indicating that perhaps a tertiary structural change has occurred at alkaline pH.

Discussion

As was previously mentioned the analysis of the dependence of number-average molecular weight on concentration yielded linear plots of m_1 *vs.* m/m_1, indicating no significant association beyond the dimer in this concentration range. Osmotic pressures were determined up to 10 mg/ml at pH 6 in an attempt to discover if higher associated forms exist at higher concentrations, but again no deviation from linearity was observed. This is in minor disagreement with the work of Millar *et al.* (1969) who found that a small amount of trimer participated in the reversible equilibrium. These authors found a dimer plus monomer association constant of 4.71×10^2 at pH 8.35. However their monomer $\rightleftharpoons$ dimer association constant at that pH, 2.85×10^3, shows rather good agreement with our value at pH 7, 3.65×10^3.

The dependence of the association on pH and ionic strength reported here still does not account for the slightly higher molecular weight reported for this protein by Birk *et al.* (1963). No attempt has been made to estimate the reflection coefficient of Staverman (1951) for solute permeation. However, the authors feel that in restricting the data to runs in which permeation did

TABLE I: Monomer ⇌ Dimer Equilibrium Constants and Free Energies for the Bowman-Birk Inhibitor under Various Solution Conditions.

pH	Temp (°C)	Ionic Strength	Calcd Value of Equil Constant × 10^{-3}	ΔF (kcal)
7	25	0.1	3.65	−4.85
	25	1.0	7.97	−5.32
6	25	0.1	10.6	−5.48
5	25	0.1	17.3	−5.77
	5	0.1	18.6	−5.41
3	25	0.1	5.14	−5.06

not exceed a rate of 8%/hr, the magnitude of the error due to permeation has been kept small. In any event the error due to the permeation effect would give molecular weights which would be too high, not too low.

As is seen in Figure 1 the association constant roughly doubles for a tenfold increase in ionic strength. This ionic strength effect is not large and can probably be attributed to the decrease in electrostatic repulsions caused by the shielding effect of the added salt. The effect of pH change on the association (Figure 2) is such that association increases as pH decreases until the isoelectric point (4.2; Birk *et al.*, 1963) is reached, after which the opposite effect is observed.

One of the possible factors influencing the variation in free energy of association with pH is the variation in the electrostatic free energy of repulsion with the change in the net charge of the monomers. An attempt can be made to quantitate this effect by use of the theory of Verwey and Overbeek (1948). The net charge on the protein at a given pH is taken as the difference between the number of protons dissociated at that pH and the isoelectric pH, the number of protons dissociated being obtained from the titration curve (Figure 3). The monomer subunit is approximated by a uniformly charged sphere, whose radius (13 Å) was obtained from the monomer molecular weight and density.

The net charge at a particular pH allows the calculation of the surface charge density, σ.

$$\sigma = \frac{q}{4\pi a_1^2} \tag{6}$$

when q is the charge and a_1 the radius of the sphere. The surface potential, ψ^0, is then approximately calculated from the Debye–Hückel theory, which gives

$$\psi^0 = \frac{4\pi a_1^2 \sigma}{D}\left(\frac{1}{a_1} - \frac{K}{1 + Ka_2}\right) \tag{7}$$

where D is the dielectric constant, K is the Debye–Hückel constant for a uni-univalent electrolyte, and a_2 is the sum of the radii of monomer and bound counter ion. Verwey and Overbeek (1948) tabulated the electrostatic potential energy of repulsion for two spheres as a function of particle separation and Ka. Assuming constant charge the electrostatic work required to bring two subunits into contact is, from their tables,

$$V = 0.39\, Da_1(\psi^0)^2 \tag{8}$$

TABLE II: Comparison of Experimental and Theoretical Changes in Free Energy with Change in Monomer Charge.

pH[a]	Charge	ΔF (kcal)	Exptl ΔF Change (kcal)	Theoretical Electrostatic ΔF Change (kcal)
7 (1.0)	−4.6	−5.32		
μ			0.47	0.40
7 (0.1)	−4.6	−4.85		
μ			0.63	0.054
6 (0.1)	−4.2	−5.48		
μ			0.29	0.356
5 (0.1)	−2.4	−5.77		
μ			0.71	0.794
3 (0.1)	+4.8	−5.06		

[a] The ionic strength is indicated in parentheses.

The last column of Table II represents the differences in the theoretical electrostatic free energy (in kilocalories per mole) between the pH values of interest.

As can be seen from Table II the effect of increasing ionic strength on the association constant is consistent with the electrostatic effect. Below pH 6 the change in association constant with pH is also consistent with the electrostatic effect. However, the experimental change between pH 6 and 7 is ten times the theoretical expected change. One possible explanation is that perhaps the single histidine residue that would be titrated in this pH region affects the association in a manner anomalous to that predicted by the idealized model used in the theoretical calculation. Otherwise, the results indicate that the electrostatic free energy of association is adequate to account for the changes in the observed free energies of association.

The apparent zero enthalpy of association is not unusual for protein association reactions. Formally, it suggests that the contribution to the enthalpy of association from the formation or breakage of hydrogen or hydrophobic bonds is either absent or cancelled by other factors. The large positive entropy can, in part, be perhaps attributed to a release of bound water on association.

While self-association is not a general property of proteinase inhibitors and the Kunitz and pancreatic inhibitors, for example, do not associate, it is possible that many do interact. The recent work of Hochstrasser *et al.* (1969), who demonstrated that trypsin inhibitors from a number of plant sources exist in polymeric forms, is indicative that such interactions may be quite common. This fact should be recognized when attempting molecular weight characterization of these species.

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Substrate Specificity at the Alkane Binding Sites of Hemoglobin and Myoglobin*

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ABSTRACT: Binding of the ligands (L) iodobutane, pentane, neopentane, butane, and xenon, to ferrihemoglobin and ferrimyoglobin, and of pentane to oxy- and deoxyhemo- and myoglobin (H is one subunit), at pH 7, 0°, is accurately described by two equilibria, $HL = H + L$, $HL_2 = HL + L$, with dissociation constants K_1 and K_2, as found earlier for β-lactoglobulin. Apomyoglobin requires three. The constants vary (*e.g.*, for pentane and β-lactoglobulin, apomyoglobin, oxyhemoglobin, oxymyoglobin, K_1 = 0.12, 0.28, 0.34, 0.8, all $\times 10^{-3}$ M). K_2 may approach $4K_1$ (*e.g.*, xenon and butane) or may be very large (*e.g.*, ferrihemoglobin pentane, and iodobutane; ferrimyoglobin and iodobutane). The ratios of K_1 for different ligands and a given protein roughly approximate the ratios calculated from partition between water and dodecyl sulfate micelles (pentane:neopentane:iodobutane:butane:xenon = 1:1.7:2:3.8:120). An exception is neopentane, which binds moderately well to ferrihemoglobin, but is almost excluded from β-lactoglobulin and ferrimyoglobin. The xenon constants fall in the expected range. In both ferrihemoglobin and ferrimyoglobin, pentane and xenon compete for the same two interacting sites. It is concluded that each protein has a single, localized, but not strictly discrete, hydrophobic binding region (*e.g.*, a circle has localized but continuous binding sites for two semicircles), which probably correspond to the xenon loci of ferrihemoglobin and ferrimyoglobin described by Schoenborn.

In early studies of alkane binding to hemoglobin (Wishnia, 1962) there appeared to be differences in the affinity of sodium dodecyl sulfate, bovine serum albumin, and Hb[1] for butane and propane suggestive of a size limitation, if not discrete binding, in Hb. In subseqeunt work the binding region of bovine serum albumin was shown to be large (Wishnia and Pinder, 1964), while βLG proved to have a small discrete site (Wishnia, 1964; Wishnia and Pinder, 1966a). When improvements in the technique promised both feasibility and precision, the study of the functional derivatives of hemoglobin (Hb, HbO_2, and Hb^+) was taken up again, in the hope that one of two possibilities would occur: (1) that binding would be to the interfaces between subunits as in bovine serum albumin, and would therefore be very sensitive to the state of the heme (Perutz *et al.*, 1968), even if a bit nonspecific with respect to alkane; the Mb derivatives would then serve as null binding controls. (2) Binding to Hb and Mb would occur at the same site in the structurally similar molecules—the Mb derivatives would be controls for the existence or nonexistence of interaction between subunits somewhat as βLGA monomer was a standard for βLGA dimer and octamer, and estimates of the strength and range of the internal stresses that accompany oxygenation could be made.

When binding proved to be discrete, a series of ligands,

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[1] Abbreviations used are: βLG, β-lactoglobulin; Hb, deoxyhemoglobin; Hb^+, ferrihemoglobin; Hb^+CN^-, ferrihemoglobin cyanide; HbO_2, oxyhemoglobin; Mb, deoxymyoglobin; Mb^+, ferrimyoglobin; Mb^+CN^-, ferrimyoglobin cyanide; MbO_2, oxymyoglobin; ApoMb, apomyoglobin.