Plurality of Pressure-Denatured Forms in Chymotrypsinogen and Lysozyme†

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ABSTRACT: The ultraviolet fluorescence of lysozyme shows a two-step decrease in efficiency when the pressure of the solution is raised from 10^{-3} to 11 kbars. The fluorescence of chymotrypsinogen decreases smoothly in a single step confined to the region below 8 kbars, with a midpoint at 5.8 kbars. At 11 kbars, lysozyme binds 1 mol of 8-anilino-1-naphthalenesulfonate (ANS) with a dissociation constant = 4.8 μM. Chymotrypsinogen binds 2 mol with a dissociation constant = 14 μM and Hill coefficient close to unity. In both proteins, binding of ANS is confined to the region above 6 kbars. In lysozyme it roughly corresponds to the second step of ultraviolet fluorescence changes. In chymotrypsinogen it has only minimal overlap with the region of protein-fluorescence change. The existence of two distinct regions of change of ultraviolet fluorescence in lysozyme and the disparity of the regions of change of protein fluorescence and of ANS binding in the two cases demonstrate the existence of a plurality of pressure-denatured forms in both proteins. On addition of tri-*N*-acetyl-D-glucosamine ((*N*-Ac-GlcN)$_3$) to lysozyme, the two-step change of fluorescence with pressure is replaced by a smooth change in a single step with a midpoint at 5.3 kbars. Analysis of the reciprocal effects expected between ligand binding and denaturation indicates that (*N*-Ac-GlcN)$_3$ binds preferentially to the denatured form of the protein. The standard free energy of binding to this form is estimated to be some 3 kcal/mol higher than the free energy of binding to the native enzyme. Computation of the spectroscopic effects expected in a protein with independent pressure-denaturable domains shows that an apparent one-step change will be observed in all but extreme cases and that the volume changes calculated from the experimental data can grossly underestimate the change in volume upon denaturation of the whole protein.

In a recent paper (Li et al., 1976), we have demonstrated the usefulness of the fluorescence techniques in the study of the pressure denaturation of proteins in solution. Our observations involved measurements of the ultraviolet fluorescence spectrum and fluorescence yield of the protein, as well as characterization of a ligand–protein equilibrium by measurements of the fluorescence of the ligand. Both methods have been used here in a study of the pressure effects upon solutions of chymotrypsinogen and lysozyme. The ligand used in these experiments was 8-anilino-1-naphthalenesulfonate (ANS[1]). By the use of these methods, we can arrive at the unequivocal conclusion that in these two proteins pressure denaturation is not a simple all-or-none transition in protein conformation but a far more complex phenomenon involving a plurality of processes. We describe first the experiments performed, which clearly disprove the two-state hypothesis. We then interpret our results with the help of a phenomenological theory that regards the protein as made up of two or more independently denaturable domains. Finally, we examine the reciprocity relations between ligand binding and domain stability and apply them to the interpretation of the findings on pressure denaturation of lysozyme in the absence and presence of a substrate (*N*-Ac-GlcN)$_3$.

Materials and Methods

Chemicals. Salt-free egg-white lysozyme (two times crystallized) and bovine chymotrypsinogen A (five times crystallized) were obtained from Worthington Biochemical Corp. and used without additional purification. The molecular weights of lysozyme and chymotrypsinogen were taken as equal to 14 400 and 25 000, respectively. The protein concentration was determined from the ultraviolet absorbancy values of 26.5 (lysozyme) and 20.4 (chymotrypsinogen) assumed for 1% protein solutions. 8-Anilino-1-naphthalenesulfonate (ANS) was the magnesium salt prepared from Eastman Kodak 8-anilino-1-naphthalenesulfonic acid as described by Weber and Young (1964). Concentration of ANS was determined by measuring the absorption at 350 nm, using an extinction coefficient of 6300 M^{-1} cm^{-1} (Kolb and Weber, 1975). Tri-*N*-acetyl-D-glucosamine ((*N*-Ac-GlcN)$_3$) was a generous gift from Dr. John A. Rupley, University of Arizona. This β-1,4-glucosamine trimer was isolated as described by Rupley (1964).

Apparatus. Optical densities were measured with a Zeiss (PMQ II) spectrophotometer. Technical fluorescence spectra at 1 atm were obtained using a photon-counting spectrofluorometer (Jameson et al., 1976). Emission spectra at high pressures were recorded on an emission spectrometer, the design of which has been published (Drotning and Drickamer, 1976). The high pressure fluorescence cell has been described by Okamoto (1974). All measurements were done at room temperature (23 °C).

Determinations of Binding Constant and Stoichiometry. The dissociation constant of the complexes of the pressure-denatured protein with ANS, K, and the number of ANS binding sites per molecule of protein, N, were determined according to the procedure suggested by Weber and Young (1964). The fraction of ANS bound, x, is related to the total protein concentration, P_0, and the total ANS concentration, X_0, by the equation:

$$x = F/F_0 = NP_0/(X_0 + K) - [K/(X_0 + K)](x^2 + x^3 \ldots) \quad (1)$$

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; (*N*-Ac-GlcN)$_3$, *N*-triacetyl-D-glucosamine.

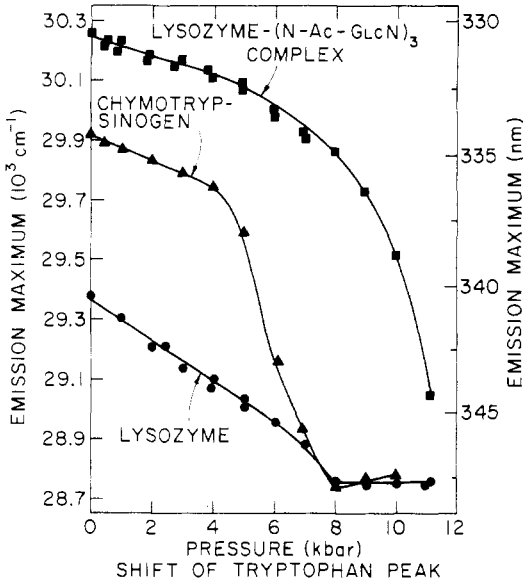


FIGURE 1: Emission band shifts vs. pressure. (▲) Chymotrypsinogen (1.6 μM); (●) 3.1 μM lysozyme; (■) 4 μM lysozyme in the presence of 1.2 mM (N-Ac-GlcN)$_3$. All solutions were buffered at a pH of 7.6 with Tris-HCl at a concentration of 0.05 M. Excitation light was at 295 nm and the temperature was 23 °C.

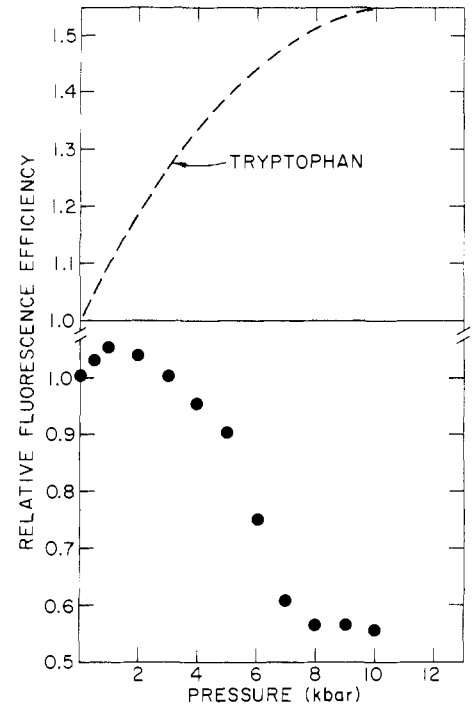


FIGURE 2: Pressure dependence of the relative fluorescence yield of chymotrypsinogen (●) and 2.4×10^{-5} M L-tryptophan (- - -). Conditions as in Figure 1.

where F is the observed fluorescence yield of ANS accompanying the binding of dye and F_0 is the fluorescence yield at the same pressure when all the dye in solution has been bound. Typically, solutions with constant ANS concentration, X_0, and increasing total protein concentrations, P_0, were separately prepared. For each solution, the fluorescence of ANS was measured as a function of pressure in the range of 1 atm to 11 kbars and the reversibility of the fluorescence signal upon release of pressure was checked. The fluorescence of totally bound ligand, F_0, was not determined experimentally, but was obtained by extrapolation to $1/P_0 = 0$ from a plot of fluorescence yield, F vs. $1/P_0$. For x small in comparison with unity, a plot of x vs. P_0 gives an initial slope, $N/(X_0 + K)$. By using $X_0 > K$, N can be determined by setting the initial slope equal to N/X_0. Once N is known, K can be determined from the relation

$$K = (NP_0/x) - X_0 \qquad (2)$$

for small values of x.

Results and Discussion

Effect of Pressure on Chymotrypsinogen. The emission maximum of tryptophan residues in native proteins commonly ranges from ca. 325 to 350 nm (30 770–28 570 cm^{-1}). Denaturation of protein by urea, which causes unfolding of their globular structures, gives rise to spectral red shifts of the maxima to the range of 350–353 nm or 28 570–28 330 cm^{-1} (Teale, 1960). Chymotrypsinogen A contains 8 tryptophans. With exciting light at 295 nm, the fluorescence spectrum of the native protein is essentially due to the tryptophans and has an emission maximum at 334 nm (29 940 cm^{-1}). Upon application of pressure, the fluorescence spectrum shifts slowly to 336 nm (29 750 cm^{-1}) at 4 kbars (Figure 1). Between 4 and 8 kbars, the spectral shift is more abrupt and from 8 to 10 kbars the emission has a maximum at 348 nm (28 750 cm^{-1}). The spectral shift is consistent with the proposition that the tryptophan residues become in contact with a more polar environment (Weber and Teale, 1965; Burstein et al., 1973). This red-shift phenomenon has been previously observed in the pressure denaturation of the riboflavin binding protein (Li et al., 1976). Figure 2 shows the relative fluorescence yield of chymotrypsinogen as a function of pressure. At pressures below the onset of denaturation (2 kbars), the fluorescence yield rises in a way similar to that observed with free tryptophan. From 2 to 8 kbars, the fluorescence yield decreases with pressure and levels off at 8 kbars at a value equal to 55% of the value at 1 atm, indicating that the process is then essentially completed. In all studies reported, reversibility was always checked by measurements at 2 pressures upon lowering the pressure on the samples. The plots of fluorescence yield against pressure can be analyzed in terms of a reversible transformation of the protein from the native to the denatured state with an equilibrium constant, k_p. The smooth decrease of fluorescence yield would lead us to apply eq 20 below, which assumes such a single-step change. From a plot of the experimental data, according to this equation two parameters are obtained: the characteristic pressure, $p_{1/2}$(exptl), at which the denaturation has proceeded halfway, and the volume change, ΔV(exptl), in the pressure-dependent reaction. The values thus calculated are shown in Table I.

TABLE I: Volume Change upon Denaturation, ΔV(exptl), and Transition Midpoint Pressure, $p_{1/2}$(exptl), from the Ultraviolet Fluorescence Observation of Proteins.[a]

Protein	ΔV(exptl) (ml/mmol)	$p_{1/2}$(exptl) (kbars)
Chymotrypsinogen	−31.2	5.8
Lysozyme (1st stage)	−19.7	4.3
Lysozyme-(N-Ac-GlcN)$_3$ complex	−25	5.3

[a] Conditions as in Figure 1.

Effect of Pressure on Lysozyme. Sophianopoulos et al. (1964) have shown that association of lysozyme into dimeric species can be easily observed in solution. The concentration

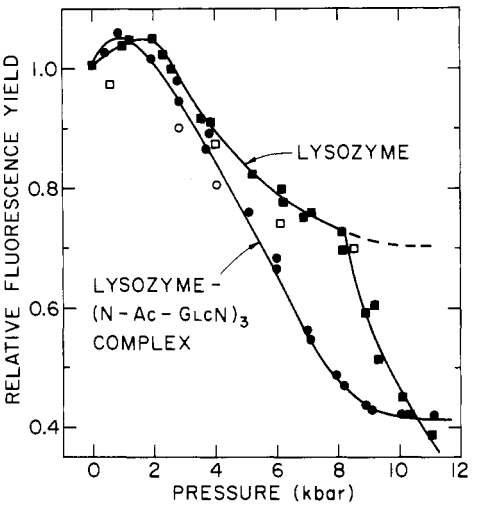


FIGURE 3: Pressure dependence of the relative fluorescence yield of lysozyme (■) and lysozyme-(N-Ac-GlcN)$_3$ complex (●). Open symbols were data obtained upon lowering the pressure of the samples. Conditions as in Figure 1. At 1 atm, the lysozyme-substrate complex has a relative fluorescence equal to 1.4 of the value for lysozyme.

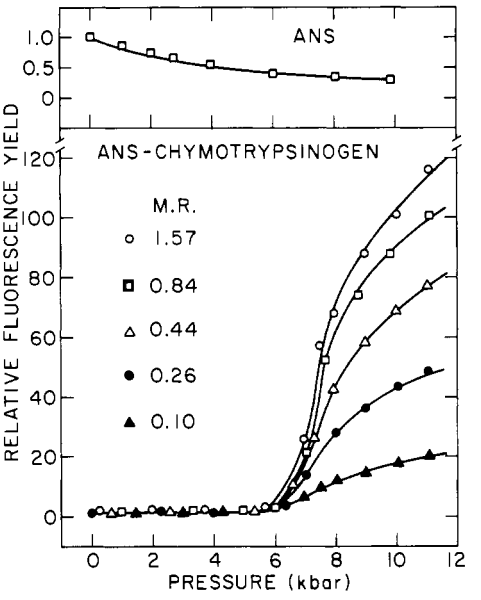


FIGURE 4: Relative fluorescence yield vs. pressure. (○) ANS (20.3 μM) + 32 μM chymotrypsinogen; (□) 20.3 μM ANS + 17 μM chymotrypsinogen; (△) 20.3 μM ANS + 9 μM chymotrypsinogen; (●) 20.3 μM ANS + 5 μM chymotrypsinogen; (▲) 20.3 μM ANS + 2 μM chymotrypsinogen. M.R.: molar ratio of chymotrypsinogen to ANS. Conditions as in Figure 4 (□) 20.3 μM free ANS.

of the protein solution thus becomes critical in a denaturation study, if dimerization is to be avoided. Our fluorimetric observations permit the use of lysozyme solutions in the micromolar range (ca. 0.004%) at which essentially no dimer is formed. The effect of pressure on the spectral emission of lysozyme is similar to that observed for chymotrypsinogen. A reversible spectral shift of emission maximum from 340 nm (29 400 cm^{-1}) at 1 atm to 348 nm (28 700 cm^{-1}) at 11 kbars is detected (Figure 1). Figure 3 illustrates the pressure dependence of the relative fluorescence yield of lysozyme. In striking contrast to chymotrypsinogen in which the denaturation curve exhibits a single step, the curve for lysozyme shows two distinct stages. A progressive decrease of fluorescence occurs from 2 to 8 kbars, followed by an abrupt fall from 8 to 11 kbars. The fluorescence decrease is substantially reversible. The two stages of the denaturation curve suggest that the pressure-induced conformational changes are localized on certain portions or domains of the protein molecules and that these changes evidently occur over different pressure ranges.

Effect of Pressure on the Lysozyme-Substrate Complex. Rupley (1964) demonstrated that the trimer of N-acetyl-D-glucosamine ((N-Ac-GlcN)$_3$), a substrate for lysozyme, is hydrolyzed only slowly at low enzyme concentrations. The extent of cleavage is negligibly small within 3 h, which is the average time required in a high-pressure fluorescence experiment. The fluorescence properties of the enzyme in the presence of the trimer have been studied by Lehrer and Fasman (1966). Upon binding to the substrate, there is an enhancement of the emission spectrum, accompanied by a blue shift of the emission maximum of 10 nm or 880 cm^{-1} (Figure 1). This is indicative of decreased polarity in the environment of either or both Try-62 and -108, which, according to Imoto et al. (1971), are responsible for the bulk of the ultraviolet emission. At the concentration of (N-Ac-GlcN)$_3$ used in our experiments, the saturation of the substrate site (Lehrer and Fasman, 1966) was over 99%.

Figure 3 shows the pressure dependence of the fluorescence of the lysozyme-substrate complex under these conditions. Remarkably, a single-step curve, similar in general shape to the one observed in chymotrypsinogen, is found. The effect of pressure on the emission maximum of the lysozyme-substrate complex is shown in Figure 1. The red shift from 330 nm (30 300 cm^{-1}) at 1 atm to 344 nm (29 100 cm^{-1}) at 11 kbars is again indicative of denaturation. At 11 kbars, the emission maxima of the enzyme (348 nm or 28 700 cm^{-1}) and the enzyme-substrate complex (344 nm or 29 100 cm^{-1}) are significantly different. This difference suggests that the tryptophan residues of the enzyme-substrate complex at 11 kbars are still in contact with an environment less polar than water. Although pressure will undoubtedly affect the stability of the complex, one can infer from the smaller blue shift at 11 kbars that the substrate is still attached to the enzyme. Table I summarizes the volume change upon denaturation, ΔV(exptl), and the midpoint pressure of transition $p_{1/2}$(exptl) for lysozyme and the lysozyme-substrate complex.

ANS-Protein Interactions at High Pressure: Effect of Pressure upon Free ANS. ANS in aqueous solution has a quantum yield of ca. 0.004 and emission maximum at ca. 520 nm (19 200 cm^{-1}). At 10 kbars, ANS shows only a small displacement (ca. 250 cm^{-1}) in the emission maximum towards longer wavelength. The relative fluorescence yield decreased with pressure and reached 0.25 of the original at 10 kbars (Figure 4).

Binding of ANS to Chymotrypsinogen under Pressure. ANS is bound by denatured proteins (Weber and Laurence, 1954) and a few native proteins (Weber and Young, 1964; Stryer, 1965) at micromolar concentration. Binding is followed by an increase in fluorescence efficiency that may reach several 100-fold. Native chymotrypsinogen at neutral pH has very low binding affinity for ANS, as measured by changes in fluorescence intensity and emission maximum. The relative fluorescence yield of 20 μM ANS at atmospheric pressure is practically unaltered in the presence of 4–32 μM chymotrypsinogen (Figure 5). The fluorescence spectrum of ANS in the presence of such concentrations of chymotrypsinogen is indistinguishable from that of free ANS. Figure 4 shows the variation of the relative fluorescence yield of ANS in the presence of the dif-

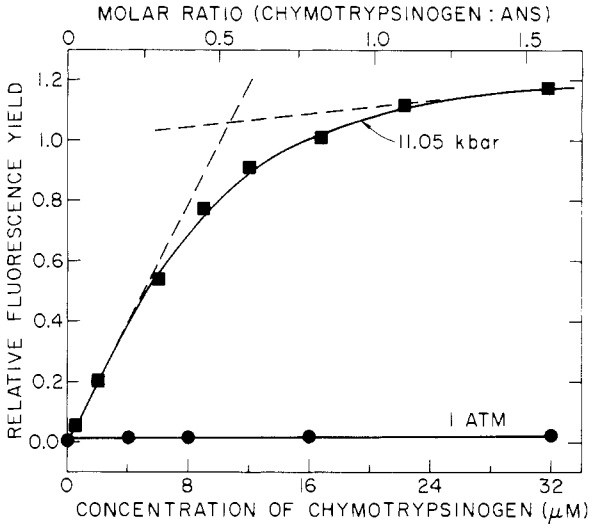


FIGURE 5: Plot of relative fluorescence yield vs. concentration of chymotrypsinogen at (●) 1 atm; (■) 11.05 kbars. Conditions as in Figure 5.

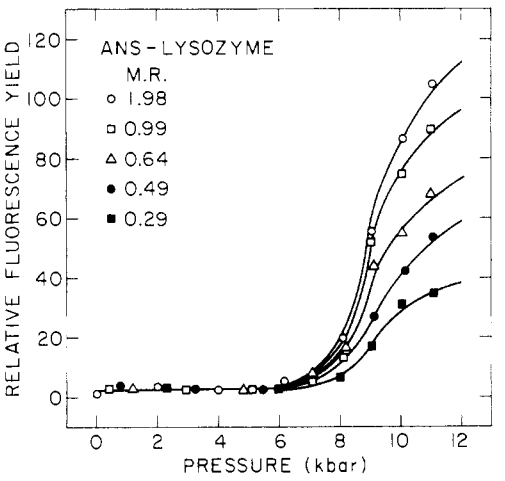


FIGURE 6: Pressure dependence of the relative fluorescence yield of 20.3 μM ANS in the presence of (○) 40.3 μM lysozyme; (□) 20.2 μM lysozyme; (△ 1) 12.9 μM lysozyme; (●) 10 μM lysozyme; (■) 6 μM lysozyme. Conditions as in Figure 4. M.R.: molar ratio of lysozyme to ANS.

ferent amounts of chymotrypsinogen. As pressure is increased from 1 atm to 6 kbars, the fluorescence yield of ANS remains insignificant. Beyond 6 kbars, contrary to the behavior of free ANS under high pressure, there is a strong fluorescence enhancement. This increases with the concentration of chymotrypsinogen and at the highest concentration employed it reaches 120 times the original value. In the range of 1 atm to 6 kbars, the emission maximum of ANS in the presence of chymotrypsinogen stays at ca. 520 nm (19 200 cm^{-1}). At 11 kbars, the emission maximum is at 484 nm (20 600 cm^{-1}). Since the chemical potential of ANS itself could be only marginally changed by the pressure, the data indicate that the conformation of the protein is being modified to facilitate the binding of ANS. Whereas observation of the ultraviolet fluorescence of the protein reveals denaturation which is essentially complete at 8 kbars (Figure 2), the fluorescence enhancement of ANS is not appreciable below 6 kbars and at 8 kbars, the fluorescence yield is still rising rapidly, implying that denaturation is still proceeding. Figure 6 illustrates the fluorescence changes when a solution of ANS is titrated with chymotrypsinogen at 11.05 kbars. The number (N) of ANS binding sites per chymotrypsinogen molecule is 2 ± 0.2, while the equilibrium constant of dissociation (K_D) of the ANS–chymotrypsinogen complex is 14.0 ± 1.8 × 10^{-6} M. A Hill plot of the data gave a straight line with a slope of 1.06, indicating equal and independent binding strengths for the two ANS sites on chymotrypsinogen.

Binding of ANS to Lysozyme under Pressure. Lysozyme is known to contain a number of different sites which can accommodate a large variety of ligands unrelated to its substrate. Thus, the enzyme has been shown to form complexes with a variety of organic molecules, such as aminostilbenes (Takenaka and Shibata, 1969). However, in 20 μM ANS solution, there was no evidence of binding of ANS by the protein, as judged by the changes in the spectrum and fluorescence intensity of ANS upon addition of the enzyme. Under high pressure, the fluorescence properties of ANS in the presence of lysozyme are analogous to those observed for the ANS–chymotrypsinogen complex. While there is little change of fluorescence yield in the pressure range of 1 atm to 6 kbars, the fluorescence increase may reach 100-fold when pressure is raised from 6 to 11 kbars (Figure 6). The emission maximum in the range of 1 atm to 6 kbars is at 520 nm (19 200 cm^{-1}). It starts to shift as pressure is higher than 6 kbars and at 11 kbars it is located at 498 nm (20 080 cm^{-1}). It is of interest to note again that the spectral shift and the fluorescence enhancement (Figure 6) of ANS in the presence of lysozyme occur after 6 kbars, while the red shift (Figure 1) and the decrease of the fluorescence of the enzyme (Figure 3) are conspicuous at much lower pressures. Whereas the tryptophanyl fluorescence observation indicates a denaturation region with $p_{1/2}$ at 4.3 kbars, the ANS fluorescence enhancement corresponds to $p_{1/2}$ = 9.6 ± 0.4 kbars. The titration curve for the binding of ANS to lysozyme at 11.05 kbars is similar to that shown for lysozyme (Figure 5). The number of ANS binding sites per molecule is 1 ± 0.1, while the equilibrium constant of dissociation of the ANS–lysozyme complex is 4.8 ± 0.8 × 10^{-6} M. Thus, the equilibrium constants for the binding of ANS to lysozyme and chymotrypsinogen are similar in magnitude, indicating that the binding forces involved must have similarities. However, the complexes are spectroscopically distinct: lysozyme–ANS has an emission maximum at 498 nm, chymotrypsinogen–ANS at 484 nm. The number of ANS binding sites seems roughly in accord with the molecular weights of the proteins, which, in turn, are directly related to their accessible surface area upon unfolding (Chothia, 1975), but the small number of ANS molecules bound and the differences in fluorescence emission of ANS in the two proteins strongly suggest that the pressure-denatured forms responsible for binding are not wholly disorganized. In thermal denaturation, proteins often form aggregates which have been shown to bind a variety of dyes usually without a definite stoichiometry. Such binding is not reversible, at least in the majority of the cases. Here the reversible effect of pressure and the definite stoichiometry indicate that we are dealing with a different phenomenon.

Chemical Equilibrium of Protein and ANS under Pressure. In contradistinction with the case of flavin and flavin binding protein (Li et al., 1976), here the ligand is virtually nonfluorescent and the denatured protein–ligand complex has a high fluorescence efficiency. If D stands for the denatured form or forms of the protein with high affinity for ANS, we have

$$q_X \ll q_{DX}; \quad q_{NX}[NX] = 0 \qquad (3)$$

where q_X, q_{DX}, and q_{NX} are the fluorescence yield of free ligand, denatured protein–ligand complex, and native protein–ligand complex, respectively. With these premises, the fraction

TABLE II: Volume Change upon Denaturation, ΔV(exptl), Transition Midpoint Pressure, $p_{1/2}$(exptl), and Volume Change upon Dissociation of Protein–ANS Complex from the Visible Fluorescence Observation of ANS in the Presence of Proteins.[a]

Protein	ΔV(exptl) (ml/mol)	$p_{1/2}$(exptl) (kbars)	Change in Volume upon Dissociation of DX Complex (ml/mol)
Chymotrypsinogen	−29.4	7.3 ± 0.3	+6.2[b]
Lysozyme	−26.2	9.6 ± 0.4	+3.1

[a] Conditions as in Figure 4. [b] Corresponds to 1 mol of protein:2 mol of ANS.

of ligand bound, γ, is given by

$$\gamma = F/F_\infty = [DX]/[X_0] \quad (4)$$

where F_∞ is the fluorescence when all the ANS is bound to the protein. This cannot be determined experimentally, but was obtained by extrapolating a plot of F vs. 1/[protein] to 1/[protein] = 0. The equilibria governing the formation of the ANS–protein complex are essentially:

SCHEME I.

$$\begin{array}{ccc} & & DX \\ & & \Big\| K_D \\ X + N & \underset{k_p}{\rightleftharpoons} & D + K \end{array}$$

where $K_D = [D][X]/[DX]$ and $k_p = [D]/[N]$. With X_0 = total ligand concentration, P_0 = total protein concentration, and proceeding as done by Li et al. (1976) for the flavin case one finds

$$K_D(1 + k_p^{-1}) = X_0(\gamma - 1) - P_0(1 - \gamma^{-1}) \equiv S(\gamma) \quad (5)$$

In the pressure range of 10 to 11 kbars, we assume that most of the native protein molecules would be converted to their denatured forms. Then $k_p^{-1} = 0$ and

$$S(\gamma) = K_D = X_0(\gamma - 1) - P_0(1 - \gamma^{-1}) \quad (6)$$

A plot of ln $S(\gamma)$ vs. pressure in this range gives a slope which will permit the calculation of the change in volume upon dissociation of the DX complex. In the lower pressure range, k_p can be calculated if we assume (d ln K_D/dp) = constant. A plot of ln k_p vs. pressure gives the change in volume upon denaturation, ΔV(exptl). Since the fluorescence yield for the binding of ANS to the proteins doesn't level off at 11 kbars, the $p_{1/2}$(exptl) values cannot be determined by simple inspection of the plots. They were determined as follows. Equation 5 may be written

$$\gamma^2 - \gamma[(1 + P_0/X_0 + K_D/X_0(1 + k_p^{-1})] + P_0/X_0 = 0 \quad (7)$$

where K_D at 11 kbars has been previously obtained and, using the experimental values of P_0 and X_0, one can calculate γ at $p_{1/2}$ by eq 7, since $k_p = 1$ at $p = p_{1/2}$. Once γ at $p_{1/2}$ is known, $p_{1/2}$(exptl) is read off directly from a plot of γ vs. pressure. Table II summarizes the values of $p_{1/2}$(exptl) and ΔV(exptl), as well as the values of the change in volume upon dissociation of the DX complex. For chymotrypsinogen, the value of −29 ml/mol for ΔV(exptl) compares closely with the ΔV(exptl) of −31 ml/mol, which was previously obtained from the ultraviolet fluorescence observation (Table I). The $p_{1/2}$(exptl) values of 7.3 ± 0.3 kbars and 9.6 ± 0.4 kbars, however, are very different from the 5.8 and 4.3 kbars obtained previously for the tryptophan fluorescence of chymotrypsinogen and lysozyme, respectively. Since k_p is related to ΔV(exptl) and $p_{1/2}$(exptl) by eq 17 below, the different values of $p_{1/2}$ are inconsistent with the two-state hypothesis. If one uses the values of $p_{1/2}$(exptl) = 7.3 and ΔV(exptl) = −29 ml/mol from the data on the ultraviolet fluorescence of chymotrypsinogen, and calculates the values of k_p as a function of pressure, eq 7 permits one to generate ANS binding curves. Figure 7 shows such computed curves of γ vs. pressure for chymotrypsinogen. Since γ is proportional to F, the computed curves can be directly compared to curves of F vs. pressure obtained experimentally (Figure 4). The simulated curves are found to have similar shapes as the observed ones, except that they are strongly displaced towards lower pressures. The experimentally observed curves are therefore inconsistent with the values of k_p generated by the observations of ultraviolet fluorescence. They must be caused by a similar process but operative at distinctly higher pressures.

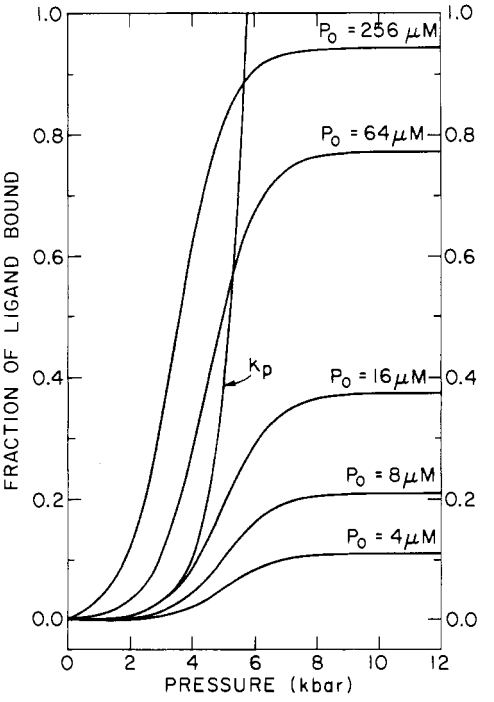


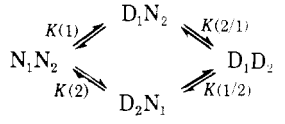
FIGURE 7: Expected ANS binding curves using the values of $p_{1/2}$(exptl) and ΔV(exptl) obtained from the ultraviolet fluorescence of chymotrypsinogen: fraction of ANS bound vs. pressure. The right ordinate shows corresponding k_p values.

Phenomenological Theory: The Effect of Pressure upon a Spectroscopic Property of a Protein with Independent Denaturable Domains. In the analysis of the changes observed in globular proteins subjected to high pressure, the existence of a unique pressure-denatured form has been generally assumed (Brandts et al., 1970; Hawley, 1971; Zipp and Kauzmann, 1973; Li et al., 1976) and the observed monotonic change of some spectroscopic variable with pressure has been taken as reasonable proof of this assumption. The experimental observations described above force us to adopt a different point of view: we assume from the start the existence of at least two separate pressure-sensitive domains within the protein, and examine the differences in the domains necessary to render experimentally evident the existence of two pressure-dependent

processes, rather than a single one. Furthermore, it seems indispensable to assess the relationships between the thermodynamic parameters deduced from the spectroscopic observations and the properties of the separate domains.

The native protein will be assumed to consist of two regions, N_1 and N_2, which undergo denaturation over two potentially different pressure ranges by conversion into the forms D_1 and D_2, respectively. The possible pressure-dependent equilibria are shown in Scheme II.

SCHEME II.



The constants in this scheme are defined by the relations:

$$K(1) = [D_1N_2]/[N_1N_2]; \quad K(2/1) = [D_1D_2]/[D_1N_2]$$
$$K(2) = [D_2N_1]/[N_1N_2]; \quad K(1/2) = [D_1D_2]/[D_2N_1] \tag{8}$$

where $K(1)$ and $K(2)$ govern the denaturation of the regions 1 and 2, respectively, when the adjoining region is still native. $K(2/1)$ and $K(1/2)$ are conditional equilibria constants (Weber, 1972) and determine the equilibria of each region when the other is already denatured. Equation 8 yields the conservation relations:

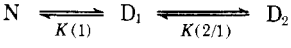
$$K(1)K(2/1) = K(2)K(1/2) = K(1)K(2)\beta \tag{9}$$

The coefficient β is related to the mutural free energy coupling of the two domains, δF°_{12} by the equation (Weber, 1975)

$$\beta = \exp(-\delta F^\circ_{12}/RT) \tag{10}$$

where $\delta F^\circ_{12} = \Delta F^\circ(2/1) - \Delta F^\circ(2)$ is the difference between conditional and unconditional standard free energy changes. When $\delta F^\circ_{12} < 0$ or $\beta > 1$ the interactions stabilize the native state of both domains. When $\delta F^\circ_{12} > 0$ or $\beta < 1$ the native states are destabilized, coupling favoring then the denatured states. Truly independent domains are characterized by $\beta = 1$. It will also be noticed that, when $K(2)$ is set equal to zero, Scheme II will also describe the progressive denaturation of the protein in successive steps according to Scheme III.

SCHEME III.



The changes in the protein subjected to pressure are inferred from observations of an average spectroscopic quantity, $\bar{a}(p)$, such as absorptivity, fluorescence efficiency, or wavelength shift. If the four species of protein of Scheme II N_1N_2, D_1N_2, D_2N_1, and D_1D_2 are present as fractions f_{11}, f_{12}, f_{21}, and f_{22} of the total protein, P_0, respectively,

$$\bar{a}(p) = f_{11}a_{11} + f_{12}a_{12} + f_{21}a_{21} + f_{22}a_{22} \tag{11}$$

In the last equation, a_{11} and a_{22} are the observable values for the protein at atmospheric pressure and at a pressure sufficiently high to convert it to the D_1D_2 form, respectively. Since

$$f_{11} + f_{12} + f_{21} + f_{22} = 1$$

then

$$\bar{a}(p) - a_{22} = f_{11}(a_{11} - a_{22}) + f_{12}(a_{12} - a_{22}) + f_{21}(a_{21} - a_{22}) \tag{12}$$

and

$$S = \frac{\bar{a}(p) - a_{22}}{a_{11} - a_{22}} = f_{11} + f_{12}\alpha_1 + f_{21}\alpha_2 \tag{13}$$

where S is the spectral value and α_1 and α_2 are, respectively, the fractions of the total change, $a_{11} - a_{22}$, contributed by the domains 1 and 2, respectively. Using the definitions of the constants in eq 8, and the last relation, one obtains

$$S = \frac{1 + \alpha_1K(1) + \alpha_2K(2)}{1 + K(1) + K(2) + \beta K(1)K(2)} \tag{14}$$

The variation of the constants K with pressure is given by the general relation

$$-\left(\frac{d \ln K}{dp}\right) = \frac{\Delta V^\circ(p)}{RT} \tag{15}$$

where $\Delta V^\circ(p)$, the standard volume change in the reaction, varies slowly with pressure owing to the difference in compressibility of the forms in equilibrium. However, to a first approximation it may be considered a pressure-independent quantity equal to the change in volume of the reaction at atmospheric pressure, ΔV°. With this assumption, the last equation takes the integrated form:

$$K = C \exp(-p\Delta V^\circ/RT) \tag{16}$$

The constant, C, may be eliminated by introduction of the pressure, $p_{1/2}$, at which $K = 1$ and eq 16 becomes

$$K = \exp[(p_{1/2} - p)\Delta V^\circ/RT] \tag{17}$$

which permits calculation of K over the whole pressure range if $p_{1/2}$ and ΔV° are given. Equations 14 and 17 show that, in general, S is a function of six parameters: β, $\Delta V^\circ(1)$, $\Delta V^\circ(2)$, $p_{1/2}(1)$, $p_{1/2}(2)$, and the ratio, m, of the fractional contributions of the domains to the spectroscopic change. For the purpose of a parametric study, we set:

$$w = \Delta V^\circ(2)/\Delta V^\circ(1)$$
$$\epsilon = p_{1/2}(2)/p_{1/2}(1) \tag{18}$$
$$m = \alpha_2/\alpha_1$$

We consider two main cases.

(1) *Independent Domains.* In this case, β is unity and the parameters subject to variation are those in eq 18. Figure 8 shows the plot of S against p for the particularly simple case in which the two domains differ only in the values of $p_{1/2}$. In this and the following figures, the region below 0.5 kbar is distorted because of the logarithmic nature of the functionality. ϵ alone has been varied, though keeping $(p_{1/2}(1) + p_{1/2}(2))/2 = p_m$ constant and equal to 5 kbars. As ϵ increases above 1, the change in S with pressure becomes less steep and for large separations of the $p_{1/2}$ values it would break up into two distinct steps. However, even in the case in which $\epsilon = 3$, it would be difficult or impossible, with the precision of today's experiments, to detect, other than a monotonic, smooth decrease of S with p. If a one-step denaturation model is then assumed by default, eq 14 gives, after setting $K(1) = K(2) = K$

$$S = (1 + K)^{-1} \tag{19}$$

and with the help of eq 17

$$\ln\left(\frac{1}{S} - 1\right) = (p - p_{1/2})\Delta V^\circ/RT \tag{20}$$

From a plot of the experimental data according to eq 20 one obtains two parameters: a characteristic pressure, $p_{1/2}$(exptl), at which $S = \frac{1}{2}$, and the apparent change in volume, ΔV(exptl) of the supposedly single domain on denaturation.

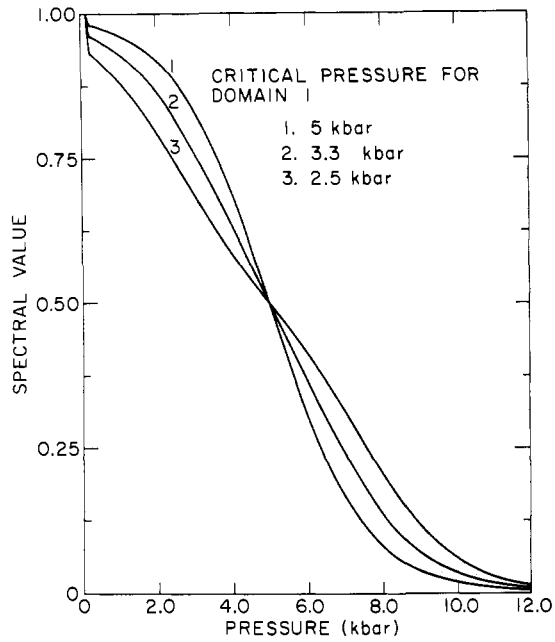


FIGURE 8: Spectral value, S, vs. pressure; $w = 1$, $\Delta V°(2) = -20$ ml/mol; $m = 1$. (1): $\epsilon = 1$, $p_{1/2}(1) = 5$ kbars; ΔV(exptl) $= -20$ ml/mol, $p_{1/2}$(exptl) $= 5$ kbars. (2): $\epsilon = 2$, $p_{1/2}(1) = 3.3$ kbars, ΔV(exptl) $= -15.5$ ml/mol, $p_{1/2}$(exptl) $= 5$ kbars. (3): $\epsilon = 3$, $p_{1/2}(1) = 2.5$ kbars; ΔV(exptl) $= -11.1$ ml/mol, $p_{1/2}$(exptl) $= 5$ kbars. Parameters used are defined in the text.

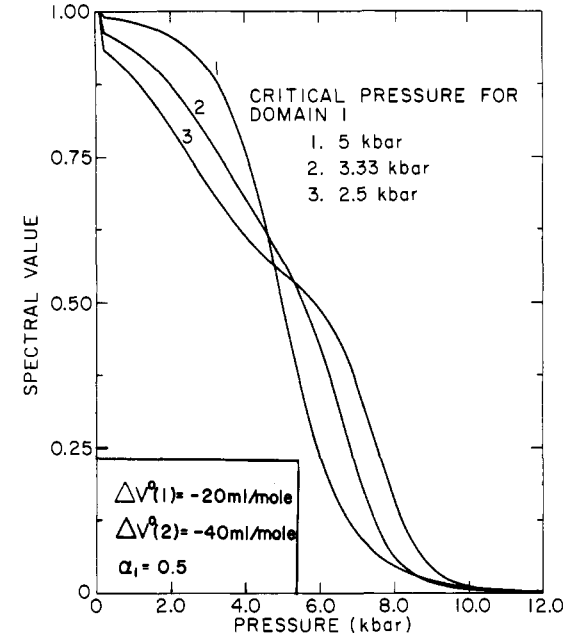


FIGURE 9: Spectral value, S, vs. pressure; $w = 2$, $\Delta V°(2) = -40$ ml/mol; $m = 1$. (1): $\epsilon = 1$, $p_{1/2}(1) = 5$ kbars; ΔV(exptl) $= -27.0$ ml/mol, $p_{1/2}$(exptl) $= 5$ kbars. (2): $\epsilon = 2$, $p_{1/2}(1) = 3.33$ kbars, ΔV(exptl) ranges from -14 to -25 ml/mol, $p_{1/2}$(exptl) $= 5.6$ kbars. (3): $\epsilon = 3$, $p_{1/2}(1) = 2.5$ kbars, ΔV(exptl) ranges from -10.4 to -23.8 ml/mol, $p_{1/2}$(exptl) $= 5.85$ kbars.

Figure 8 shows that ΔV(exptl) reaches the actual value for the domain when $\epsilon = 1$, but falls below this value as the difference between the $p_{1/2}$ values assigned to the domains increases to the point that ΔV(exptl) $\simeq \Delta V°(1)/2$ for $\epsilon = 3$. When additionally one assumes $m = 2$ or $m = \frac{1}{2}$, so that either the low pressure or the high-pressure denaturable domain, respectively, has the dominant spectroscopic contribution, monotonic curves are still obtained which do not leave much room for suspecting other than a one-step process. ΔV(exptl) decreases with increasing ϵ just as in the case $m = 1$, shown in Figure 11, and $p_{1/2}$(exptl) falls below p_m if $m > 1$, or rises above it, if $m < 1$.

Figure 9 shows the expected spectroscopic changes when both the volumes, $\Delta V°$, and the characteristic pressures, $p_{1/2}$, are different in the two domains, but the spectroscopic contributions are kept equal. In the examples in the Figure 9, $\Delta V°(2) = 2\Delta V°(1)$, and one sees that realistically recognition of a two-step process would be possible only if ϵ is as high as 2 to 3. It may be noticed that the values of ΔV(exptl) recovered from plots according to eq 20 would be smaller than the real values by about one-third. If, additionally, the spectroscopic contributions of the domains are allowed to differ, the two-step character is exaggerated, if $m < 1$, or decreased to the point of disappearance, if $m > 1$.

(2) Dependent Domains. If $\delta F°_{12} > 0$ or $\beta < 1$, the denaturation of one of the domains makes the remaining one more stable and the results are then qualitatively similar to those already discussed, there being no formal distinction between a value of $p_{1/2}(2)$ intrinsically higher than $p_{1/2}(1)$ or rendered so by the denaturation of the first domain. On the other hand, if $\delta F°_{12} < 0$ or $\beta > 1$, denaturation of the first domain facilitates the denaturation of the second and if $\beta K(2) > I(1)$ a qualitatively new effect, the cooperative denaturation of the two domains, appears. Figure 10 illustrates the situation with an example in which $\Delta V°(2) = 2\Delta V°(1)$, $p_{1/2}(2) = 3p_{1/2}(1)$ and $m = 1$. It is seen that the two-step character, apparent for $\delta F°_{12} = 0$, decreases and disappears as the absolute value of

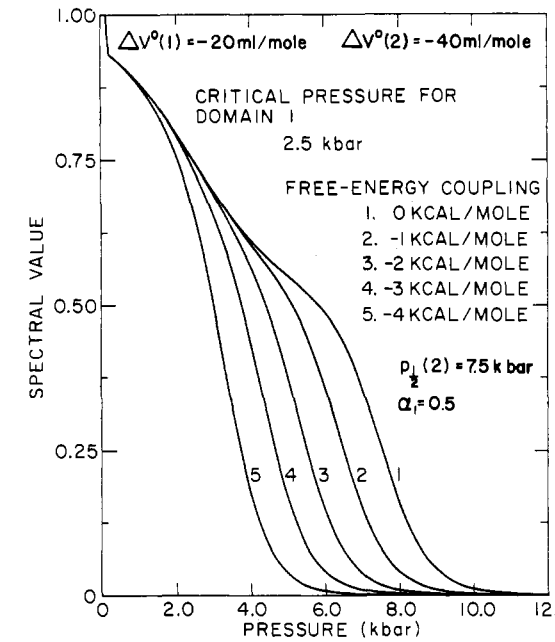


FIGURE 10: Spectral value, S, vs. pressure; $w = 2$, $\Delta V°(2) = -40$ ml/mol; $m = 1$, $\epsilon = 3$, $p_{1/2}(1) = 2.5$ kbars. (1): $\delta F°_{12} = 0$ kcal/mol. (2): $\delta F°_{12} = -1$ kcal/mol. (3): $\delta F°_{12} = -2$ kcal/mol. (4): $\delta F°_{12} = -3$ kcal/mol. (5): $\delta F°_{12} = -4$ kcal/mol.

$\delta F°_{12}$ is increased. At the same time ΔV(exptl) rises, but in all the cases shown in Figure 10

$$\Delta V(\text{exptl}) < \Delta V°(1) + \Delta V°(2) \qquad (21)$$

The important point follows that, even when an undoubtedly cooperative phenomenon is at the basis of the denaturation process, the value of ΔV(exptl) is likely to be appreciably smaller than the total volume change of the cooperative region

upon denaturation. It is only when β becomes unreasonably large that one could expect the inequality in eq 21 to disappear.

Extension of the Theory to the Case of Many Denaturable Domains. The theory sketched above may be generalized to the case of M denaturable domains within the protein. The possible chemical species are of the type: $D_J N_{M-J}$. The equilibria among them are governed by M statistical constants of the form[2]

$$K_J = \frac{J[D_J N_{M-J}]}{(M - J + 1)[D_{J-1} N_{M-J+1}]} \tag{22}$$

The equivalent of eq 13 for such system reads

$$S = f_0 + \sum_{J=1}^{M-1} f_J a_J \tag{13a}$$

where f_J is the fraction of P_0 with J denatured domains and a_J is the average fractional contribution that this species can make to the total spectroscopic change upon complete denaturation. Writing the values of f_J as functions of f_0 and of the constants K_J gives the equivalent of eq 14

$$S = \frac{1 + \sum_{J=1}^{M-1} \binom{M}{J} a_J B_J}{1 + \sum_{J=1}^{M} \binom{M}{J} B_J} \tag{14a}$$

where $B_J = K_1 K_2 \ldots K_J$. If all the K's are equal,

$$B_J = K^J \tag{23}$$

$$S = \frac{1 + \sum_{J=1}^{M-1} \binom{M}{J} a_J K^J}{(1 + K)^M} \tag{24}$$

Moreover, if the spectroscopic contributions of the domains are equal

$$a_J = \frac{M - J}{M}; \quad \binom{M}{J} a_J = \binom{M-1}{J}$$

and

$$S = \frac{(1 + K)^{M-1}}{(1 + K)^M} = (1 + K)^{-1} \tag{19a}$$

identical to eq 19. An apparently unique denaturation process will be observed in these cases with ΔV(exptl) = total $\Delta V°/M$ and, unless the number of domains is identified in some independent fashion, the real volume change of the protein upon denaturation will be grossly underestimated. The underestimation will be even worse if the domains have values of $p_{1/2}$ which are not the same, as illustrated in Figure 8 for the case of two domains.

Significance of the Spectroscopic Changes with Pressure. The analyses of the binding data for ANS (Tables I and II) indicate a decrease in volume of a tenth to a fifth of ΔV(exptl) upon formation of the protein–ANS complexes. Li et al. (1976) found that the formation of the flavin monucleotide–riboflavin binding protein complex resulted in a decrease in volume of 3.5 ml and Weber et al. (1974) found an almost equal value for the formation of the folded complex in flavin adenine dinucleotide. From these and a few similar figures in the literature, we can estimate that in the formation of noncovalent adducts of small molecules the volume change is of the order of a percent or less of the interacting parts. A similar figure has been arrived at for the "conformational volume change" that results upon folding a peptide chain (Zamyatnin, 1972). Therefore, the values of 20–30 ml/mol of ΔV(exptl) must result from conformational changes involving protein regions of a few thousand daltons and are inconsistent with any explanation that envisions the spectroscopic changes as resulting from localized effects involving only the probes, tryptophan or ANS, and their immediate surroundings. This consideration provides the main justification for referring to the cause of the spectroscopic changes under pressure as "denaturation"

The experimental data presented leave no doubt that the pressure denaturation of the two proteins studied involves several independent or consecutive processes. It does not seem realistic to postulate that the protein undergoes denaturation through a series of stable intermediates that involve the *whole* molecule, and the more natural interpretation is that the experiments reveal the existence of independently denaturable domains within the protein. Assuming that both chymotrypsinogen and lysozyme comprise only two such domains, and that the change in volume of these upon denaturation is that determined from the ultraviolet and ANS-binding spectral changes, in each case, one would estimate the total protein volume changes to be −50 to −70 ml/mol. However, there is, at present, no particular reason to believe that the number of denaturable domains is only two, and that each of them undergoes a totally cooperative denaturation reaction that would make the volume change accurately the sum of the volume changes of its component parts (eq 21). Thus, the actual volume changes accompanying the denaturation could be severalfold larger than the calculated ones. The values of critical pressures observed, $p_{1/2}$(exptl), depend not only on the number and individual critical pressures of the domains, but also upon the representation of *each* domain in the change in spectral value, S. Therefore, the value of $p_{1/2}$(exptl) itself cannot be given a precise physical significance, at least at this stage of our knowledge of the number and nature of the domains. These reservations would appear to limit severely the interest of the observations of the spectroscopic changes of proteins under pressure as regards the determination of thermodynamic quantities characteristic of the whole protein. On the other hand, the demonstration that the spectral changes result from a plurality of conformational domains of substantial mass with characteristic critical denaturation pressures and volumes and ligand binding properties opens the way for a study of new and far more interesting problems relating to both function and conformation of proteins.

Effect of Ligand Binding upon the Pressure Denaturation of a Protein with Two Domains. The description of the effects is made simpler by the use of the statistical equilibrium constants already used in the derivations of the denaturation equation for M domains (eq 14a). When $M = 2$, the statistical constants K_1 and K_2 are related to the individual domain constants $K(1) \ldots K(2/1) \ldots$ of eq 8 by the relations

$$K_1 = \exp(-\Delta F°_1/RT);$$
$$\Delta F°_1 = (\Delta F°(1) W_1 + \Delta F°(2) W_2)/(W_1 + W_2) \tag{25}$$

$$K_2 = \exp(-\Delta F°_2/RT);$$
$$\Delta F°_2 = (\Delta F°(2/1) W_1 + \Delta F°(1/2) W_2)/(W_1 + W_2) \tag{26}$$

where

$$W_1 = \exp(-\Delta F°(1)/RT); \quad W_2 = \exp(-\Delta F°(2)/RT) \tag{27}$$

[2] The statistical constants, K_J of eq 22 are not equivalent to $K(1)$, $K(2)$, . . . etc. of eq 8, which are nonstatistical constants belonging to individual domains. These are useful in analyzing in detail the simple case of a protein with two domains, but their explicit consideration would unnecessarily complicate the general case of many domains.

and the $\Delta F°$'s are, alternatively, the statistical, or the individual, standard free energies of denaturation. By adopting the statistical constants, there is no need to distinguish more than three species: N (native), I (semidenatured), having one native and one denatured domain, and D (wholly denatured). We then have

$$K_1 = [I]/2[N]; \quad K_2 = 2[D]/[I] \tag{28}$$

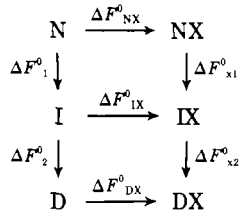
and eq 14a reads then simply

$$S = \frac{1 + 2\alpha K_1}{1 + 2K_1 + K_1K_2} \tag{29}$$

where α is the average spectral fractional change when one domain is denatured.

We denote the standard free energies of binding of the ligand X of the three species N, I, D by $\Delta F°_{NX}$, $\Delta F°_{IX}$, $\Delta F°_{DX}$, respectively. The free energy changes in the reaction N → I and I → D are, respectively, $\Delta F°_1$ and $\Delta F°_2$. Those of the reactions NX → IX and IX → DX are, respectively, $\Delta F°_{x1}$ and $\Delta F°_{x2}$. These free energies are related by Scheme IV which permits

SCHEME IV.



one to deduce the conservation relations:

$$\Delta F°_1 - \Delta F°_{x1} = \Delta F°_{NX} - \Delta F°_{IX} = \delta F°_1$$
$$\Delta F°_2 - \Delta F°_{x2} = \Delta F°_{IX} - \Delta F°_{DX} = \delta F°_2 \tag{30}$$

According to eq 30, the difference in the affinity for the ligand exhibited by the native and denatured forms in equilibrium must equal the difference in stability of the free and ligand-bound conformations. Therefore, $\delta F°_1$ and $\delta F°_2$ are the standard free energy couplings between the binding of the ligand and the denaturation reaction of the domains.

Combining eq 30 and 17 we find an expression for K_1 and K_2 when a bound ligand introduces a free-energy coupling $\delta F°$ with the denaturation reactions:

$$K_1 = [(\exp(p_{1/2} - p))(\Delta V°/RT)] \exp(-\delta F°_1/RT)$$
$$K_2 = [(\exp(p_{1/2}' - p))(\Delta V°/RT)] \exp(-\delta F°_2/RT) \tag{31}$$

$p_{1/2}$ and $p_{1/2}'$ are the characteristic pressures at which K_1 and K_2 are, respectively, unity, in the absence of coupling, and $\Delta V°$ and $\Delta V°'$ are the volume changes upon denaturation in the two stages of the reaction.

Stabilization of the Denatured Form of Lysozyme by the Binding of Tri-N-acetyl-D-glucosamine. Figure 3 shows that the main effect of addition of *N*-acetyl-D-glucosamine to lysozyme is a large shift of the second step of denaturation towards lower pressures, while the first step shows an almost insignificant shift in the same direction. The general result is the virtual disappearance of the two-step character of the pressure changes, so evident in the unbound protein. Figure 11 shows a plot of S vs. p calculated for the parameters $p_{1/2}$ = 4 kbars, $p_{1/2}'$ = 9 kbars, $\Delta V°$ = −20 ml/mol, $\Delta V°'$ = −40 ml/mol and m = 1. These parameters were chosen to obtain a profile of spectroscopic changes with pressure of the same general shape as observed for lysozyme free of substrate. Equation 31 was employed for the calculation of K_1 and K_2 at

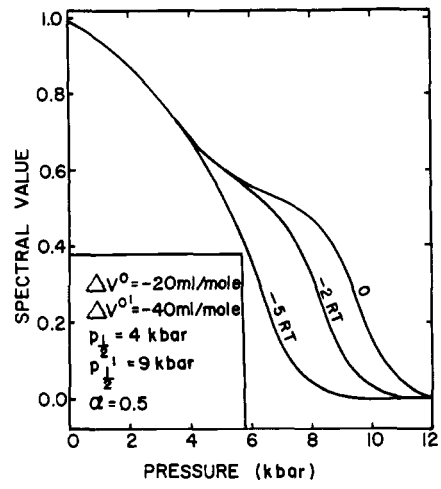


FIGURE 11: Spectral value, S, vs. pressure; $\Delta V°$ = −20 ml/mol, $\Delta V°'$ = −40 ml/mol; m = 1.

any pressure and fixed value of $\delta F°_1$ and $\delta F°_2$. It is seen in the figure that for $\delta F_1 = 0$, $\delta F_2 = -5RT$ (2.9 kcal/mol) the two-step character is replaced by a smooth change of S with p. $p_{1/2}$(exptl) is found at ~5.2 kbars and $\Delta V°$(exptl) = −24 ml/mol, values quite close to those found for lysozyme in the presence of the substrate. We conclude that (*N*-Ac-GlcN)$_3$ binds to the denatured form with a standard free-energy change that exceeds that of binding to the native form by ca. 3 kcal/mol. Superficially, this may appear as a strange conclusion because a specific conformation, characteristic of the native protein, is deemed necessary for specific substrate binding. However, it has been repeatedly suggested (Jencks, 1969; Williams, 1971) that the native enzyme binds a strained conformation of the substrate and that such strained complexes materially add to the chances of chemical reaction. In that case, particularly if pressure denaturation leads to a limited loosening of the protein structure with penetration of solvent rather than to a complete unfolding of the protein, one may often expect the substrate to bind more strongly to the pressure-denatured than to the native conformation. Clearly, these experiments suggest that pressure effects can provide a test of the strained character of enzyme–substrate complexes.

Conclusions

(1) Observations of the change in the yield of ultraviolet fluorescence emission of lysozyme with pressure permit the detection of two distinct steps of spectroscopic change covering the regions of 4–8 kbars and 8–11 kbars, respectively.

(2) Although the change in yield of ultraviolet fluorescence of chymotrypsinogen with pressure appears to consist of a single step with a midpoint at 5.8 kbars, the existence of a further denaturation process is demonstrated by the increased binding of anilinonaphthalenesulfonate in the region of 6–10 kbars but not below this. Formation of a similar lysozyme–ANS complex takes place also in this high-pressure region and roughly corresponds to the second denaturation step detected by changes of ultraviolet protein fluorescence.

(3) The complexes of ANS with the pressure-denatured proteins have definite stoichiometry and spectroscopic properties. At 11 kbars, lysozyme binds 1 mol of ANS with a dissociation constant of 4.8 μM and fluorescence emission maximum at 498 nm. Chymotrypsinogen binds 2 mol of ANS with maximum fluorescence emission at 484 nm. The binding of these 2 mol of ligand is independent, as shown by a Hill coefficient close to unity, and the common dissociation constant

is 14 μM. All the pressure-dependent changes of the fluorescence of protein and ligand were reversible within the error of the measurement.

(4) The preceding results leave no doubt that the assumption commonly used in this field for the analysis of the results, namely, that the pressure denaturation of proteins is a simple one-step, or two-state, phenomenon, is untenable. The simplest model, that of a protein with two independent denaturable domains, has been considered in detail. In the present state of the experimental art, an apparent one-step change of a spectroscopic quantity with pressure will be observed unless *both* the characteristic denaturation pressures, $p_{1/2}$, and the changes in volume upon denaturation of the two domains differ by at least a factor of two. It is noteworthy that the change in volume upon denaturation, calculated by treatment of the data as belonging to a two-state (single-step) process, does not correspond to the change in volume of the total protein but yields only the volume change of an independent domain, or a fraction of it. Accordingly, we estimate that the total volume change on denaturation in our two proteins is somewhere between -50 and -100 ml/mol, if these proteins comprise only two independent domains, and an even larger quantity if the number of independent domains is greater than two.

(5) The complex of lysozyme with tri-*N*-acetyl-glucosamine shows a single step of ultraviolet fluorescence change with pressure, with a midpoint at 5.3 kbars. The difference in behavior with that of lysozyme in the absence of $(N\text{-Ac-GlcN})_3$ arises from stabilization of the pressure-denatured protein owing to the increased strength of binding of the substrate to this as compared to the native protein. It is estimated that the difference in the standard-free energy of binding of the ligand to the two forms is close to 3 kcal/mol.

The general conclusion from our observations is that one cannot, at present, expect to obtain with certainty from the spectroscopic data the total volume change of the protein upon denaturation, or estimate the corresponding free-energy changes. Rather, the experimental values determined belong to a "denaturable domain", which needs to be defined with the help of new experimental techniques. Among these, a study of the characteristic chemical reactions of various protein groups over the whole pressure range appears to be the most promising.

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