1432.
Smith, L., and Conrad, H. (1956), *Arch. Biochem. Biophys. 63,* 403.
Smith, L., Davies, H. C., and Nava, M. (1974), *J. Biol. Chem. 249,* 2904.
Smith, L., Davies, H. C., Reichlin, M., and Margoliash, E. (1973), *J. Biol. Chem. 248,* 237.
Staudenmayer, N., Ng, S., Smith, M., and Millett, F. (1976), submitted to *Proc. Natl. Acad. Sci. U.S.A.*
Takano, T., Kallai, O. B., Swanson, R., and Dickerson, R. E. (1973), *J. Biol. Chem. 248,* 5235.
Takemori, S., and King, T. E. (1964), *J. Biol. Chem. 234,* 3546.
Wada, K., and Okunuki, K. (1969), *J. Biochem. 66,* 249.

Effects of Pressure upon the Fluorescence of the Riboflavin Binding Protein and Its Flavin Mononucleotide Complex†

Thomas M. Li, John W. Hook, III, Harry G. Drickamer, and Gregorio Weber*

ABSTRACT: The effect of pressure in the range of 10^{-3}–10 kbars upon the ultraviolet fluorescence of the riboflavin binding protein and the fluorescence of its complex with flavin mononucleotide has been studied. The fluorescence spectrum of the isolated protein showed a reversible red shift of 12 nm (1000 cm^{-1}) at high pressure, indicating the reversible exposure of the tryptophan to solvent. From the pressure dependence of the visible fluorescence of the protein–flavin complex in the region of 1–4 kbars the volume change in dissociation of the protein–ligand complex was estimated to be +3.3 ml/mol. A very sharp increase in fluorescence—up to 30-fold of the low-pressure value—takes place in the region 5–8 kbars. This increase is due to release of the flavin from the complex and is assigned to pressure denaturation of the protein. The midpoint, $p_{1/2}$, of this transition was found at 6.5 kbars and the change in volume, ΔV, in the reaction (native-to-denatured) was calculated to be −74 ml/mol. Addition of up to 30% methanol results in a progressive decrease both in ΔV and $p_{1/2}$, in agreement with the concept that hydrophobic bonding stabilizes the native structure.

The reversible pressure denaturation of proteins was first demonstrated by Brandts et al. (1970) who reported changes in the absorption of the aromatic residues of ribonuclease A subjected to pressures up to 3.4 kbars. Hawley (1971, 1975) and Zipp and Kauzmann (1973) have made similar observations on chymotrypsinogen and metmyoglogin, respectively. In all cases, the pressure-induced reversible denaturation of the protein was followed by ultraviolet or visible absorption spectrophotometry. In general fluorimetric techniques offer much higher sensitivities than absorption measurements. The lower protein concentration required for fluorescence spectroscopy permits working under practically ideal thermodynamic conditions that minimize any anomalous effects due to aggregation of protein molecules. Absorption measurements are restricted to the determination of band position and absorbancy. Fluorescence measurements can include observation of fluorescence lifetimes and polarization, which together with band position and emission intensity can provide wider information than the absorption observations. In this paper we restrict ourselves to observations of intensity and spectral distributions of the emission by the protein itself and by a specific fluorophore, flavin mononucleotide (FMN),[1] which is in binding equilibrium with the specific protein, the riboflavin binding protein (RBP) from hen's egg white. By these means we are able to characterize the changes in equilibrium between ligand and protein as pressure is raised, as well as demonstrate the phenomenon of pressure denaturation, and calculate the characteristic denaturation pressure and the change in volume in this reaction.

Materials and Methods

Riboflavin binding protein from Leghorn egg white was prepared according to Becvar (1973). Physical homogeneity of the protein was ascertained by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The dialyzed protein was stored in polycarbonate bottles and frozen at −15 °C until use. Concentrations of protein solutions were determined from the absorption coefficient of 4.9×10^4 M^{-1} cm^{-1} at 281 nm (Becvar, 1973). Flavin mononucleotide from Sigma Chemical Co. was purified on DEAE-cellulose columns according to Massey and Swoboda (1963). The FMN concentration was determined from the absorbance of 375 nm assuming an absorption coefficient of 10.4×10^3 M^{-1} cm^{-1}. L-Tryptophan was purchased from Nutritional Biochemicals. *N*-Bromosuccinimide (NBS) was obtained from the Fisher Scientific Co. and recrystallized from water before use. Methanol was spectro-quality from Matheson Coleman and Bell. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer was prepared from Trizma base (Sigma). Other chemicals used were reagent quality and were used without further purification.

Choice of Buffer. The pH of buffer solutions changes under pressure. Tris-HCl has been shown to be almost pressure insensitive (Neuman et al., 1973). The pH of a Tris-HCl buffer decreases by less than 0.2 unit over a pressure range of 6.3 ki-

† From the Department of Biochemistry (T.M.L. and G.W.), School of Chemical Sciences and Materials Research Laboratory (J.W.H. and H.G.D.), University of Illinois, Urbana, Illinois 61801. *Received February 23, 1976.* This work was supported in part by Grant 11223 of the National Institute of General Medical Sciences, United States Public Health Service, and in part by the United States Energy Research and Development Administration under Contract E(11-1)-1198.

[1] Abbreviations used are: FMN, flavin mononucleotide; RBP, riboflavin binding protein; NBS, *N*-bromosuccinimide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; AMP, adenosine 5′-monophosphate.

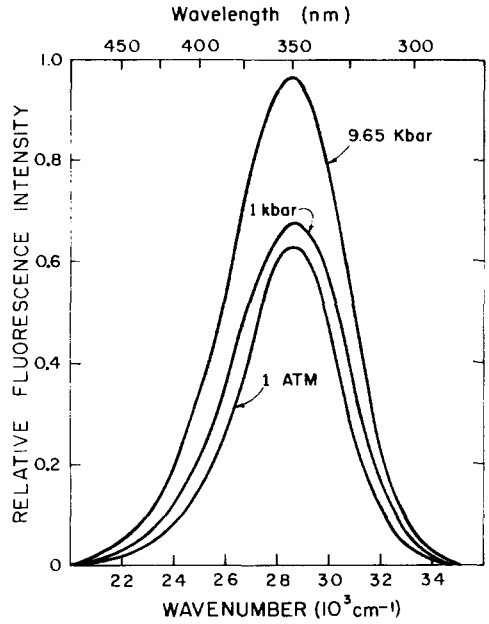

FIGURE 1: Corrected fluorescence spectra of L-tryptophan at pressures in the range of 0–10 kbars. Measurements were obtained with 2.4×10^{-5} M tryptophan at 23 °C in 0.05 M Tris-HCl buffer, pH 7.6. The excitation light was at 280 nm.

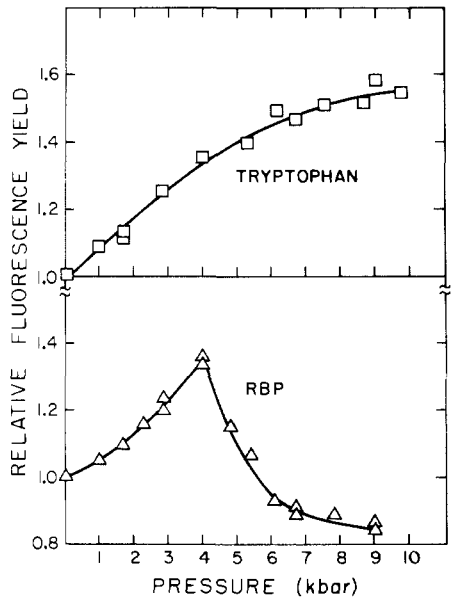

FIGURE 2: Relative fluorescence yield vs. pressure: (△) RBP, (□) L-tryptophan.

lobars and on this basis, Tris-HCl was selected for the high-pressure experiments.

Apparatus. Optical densities were measured with a Zeiss (PMQII) spectrophotometer. The oxidation of tryptophan with NBS was followed by recording the decrease in absorbance at 280 nm vs. time in a Beckman Acta M VI spectrophotometer. Fluorescence emission spectra at 1 atm were determined on the spectrofluorometer described by Weber and Young (1964).

Fluorescence Measurement at High Pressure. A high-pressure liquid cell was employed that uses spectral grade hexane as the pressure transmitting fluid (Weber et al., 1974). Pressure calibration was determined with a manganin resistance gauge. Description of the high-pressure liquid cell, the internal cell containing the sample, optical window plugs,

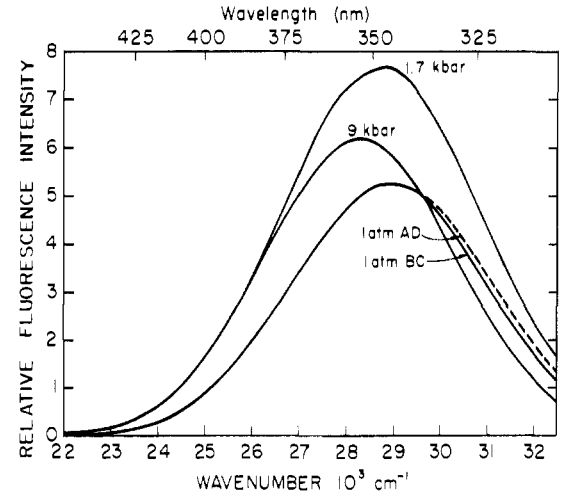

FIGURE 3: Corrected fluorescence spectra of riboflavin binding protein with 2.7×10^{-6} M RBP at 23 °C in 0.05 M Tris-HCl buffer, pH 7.6. Excitation wavelength was at 295 nm: (BC) before compression, (AD) after decompression.

calibration, and pressure intensifier has been given by Okamoto (1974).

Fluorescence emission spectra in the range of 10 kilobars were determined on the emission spectrometer described by Drotning (1975). A Hanovia 901C 150-W xenon arc lamp was used as a source of excitation. The emitted light was observed at a right angle to the excitation light. The source fluctuation was compensated by electronically dividing the emission signal by a signal proportional to the exciting light. The intensities measured at each wavelength were digitally averaged and later corrected for the transmission of the grating and the response of the photomultiplier as a function of wavelength. Relative quantum yields of fluorophores were then obtained by comparing their corrected spectra.

Results

The fluorescence spectrum of apo-riboflavin binding protein consists of one broad band with a maximum near 342 nm that may be assigned to the protein's nine tryptophan residues. Since no data were available concerning the effect of pressure on the fluorescence of tryptophan, this was measured first. Representative fluorescence spectra of tryptophan at different pressures in the range of 10 kbar are shown in Figure 1 that indicates that the emission maximum of tryptophan is not appreciably shifted by pressure (ca. 100 cm^{-1} at 10 kbars) and in Figure 2 according to which the relative fluorescence yield of tryptophan increases with the pressure up to 10 kbars.

The effect of increasing pressure on the relative fluorescence yield of RBP is also shown in Figure 2. Initially the fluorescence yield increases with increasing pressure, just as in free tryptophan, but a sharp decrease occurs at 4 kbar. The fluorescence spectra of RBP at different pressures are shown in Figure 3. At one atm, the spectrum of RBP has an emission maximum at 342 nm (29 240 cm^{-1}). A small red shift is already observed when the pressure is increased to 1.7 kbars and a much larger red shift is seen at 9 kbars, when the emission maximum is at 354 nm (28 240 cm^{-1}). Both the 12-nm (1000 cm^{-1}) red shift and the fluorescence yield changes are reversible; the fluorescence spectra at 1 atm before compression and after decompression are in excellent agreement. The red shift indicates the exposure of the tryptophan residues from the interior of the protein to a more polar environment, provided probably by the water molecules, and its magnitude

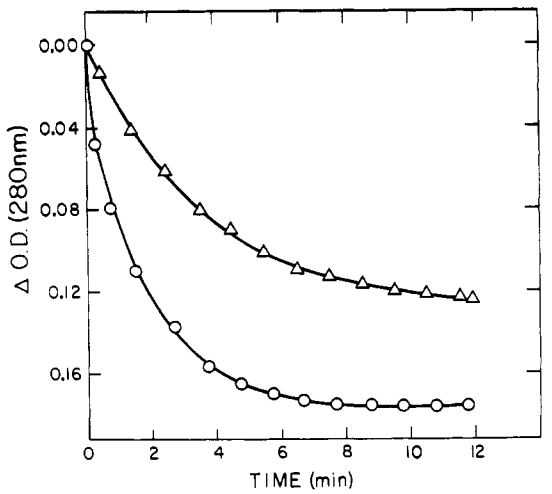

FIGURE 4: Effect of *N*-bromosuccinimide upon RBP and RBP-FMN complex. The change in optical density of RBP and RBP-FMN complex at 280 nm upon reaction with 0.85×10^{-6} M NBS was plotted against time: (○) 10×10^{-6} M RBP; (△) 10×10^{-6} M RBP in the presence of 15×10^{-6} M FMN.

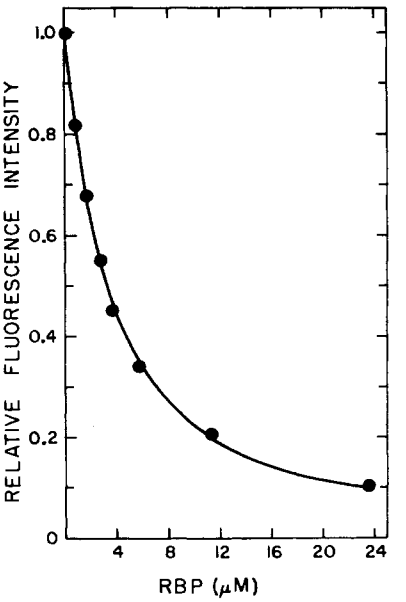

FIGURE 5: Relative fluorescence of FMN at 525 nm vs. RBP concentration in 0.05 M Tris-HCl buffer (pH 7.6) at 23 °C. Excitation wavelength was 445 nm. The concentration of FMN is 1.7×10^{-6} M. The solid line is the theoretical quenching curve expected for $K_D = 2.45 \times 10^{-6}$ M.

corresponds to a difference in the interaction energies of the ground and fluorescence state with their surroundings in the native and denatured state, respectively, of 2.8 kcal/mol of tryptophan.

Fluorescence of RBP-FMN Complexes. The riboflavin binding protein binds riboflavin very tightly at neutral pH with a dissociation constant, K_D, of 1.3×10^{-9} M and a stoichiometry of 1 to 1 (Becvar, 1973). RBP can also bind with varying affinities several flavin derivatives including FMN. It is observed that upon binding, both the fluorescence of FMN and the tryptophan fluorescence of the protein are quenched. The quenching of the protein fluorescence by FMN is the result of efficient electronic energy transfer from excited tryptophan to FMN. The protein fluorescence is completely overlapped by the strong FMN absorption and from this overlap and the fluorescence lifetime of RBP of 1.5 ns, an approximate value for the Forster's parameter R_0 of 35 Å may be calculated. A globular protein of molecular weight 32 000 has a diameter approximately of 42 Å, so that on this basis considerable quenching of tryptophan fluorescence by efficient transfer of energy to the bound FMN can be predicted. On the other hand, it has been known that many aromatic compounds quench flavin fluorescence (Weber, 1950, 1966) following formation of weak molecular complexes. Quenching of the flavin fluorescence upon binding to the protein requires direct contact with tyrosine or tryptophan residues. If the latter is the case, one would expect protection of the tryptophan against oxidation by *N*-bromosuccinimide. NBS was allowed to react with RBP. As a result of the oxidation of the tryptophan residues, the optical density at 280 nm that corresponds to the absorption of tryptophan decreased with time (Figure 4). When the experiment was repeated in the presence of a saturating amount of FMN, a smaller change of optical density was observed. The difference in the extent of oxidation, which remained after 24 h, can be used to compute the apparent number of tryptophans that are protected, using the equation (Witkop, 1961),

$$n = 1.3\Delta A_{280}/5500M_{RBP} \quad (1)$$

where ΔA_{280} is the drop in optical density at 280 nm, and M_{RBP} is the molar concentration of RBP. It is found that 1.3 tryptophans are protected. Since the NBS reaction is not entirely specific for tryptophan—tyrosine and histidine could also be attacked (Witkop, 1961)—the number of tryptophans calculated is only approximate. Probably there are one or two tryptophans at the FMN binding site.

It is observed that FMN free in solution is strongly fluorescent but when complexed with RBP the fluorescence is totally quenched. The quenching of FMN fluorescence upon addition of RBP is shown in Figure 5 that gives a plot of the relative fluorescence intensity at 525 nm vs. the concentration of RBP at neutral pH and room temperature. The emission drops progressively as protein is added and if a sufficient excess of protein is present, virtually all the FMN fluorescence can be quenched.

The binding of FMN to RBP can be represented by the reaction:

$$\text{FMN} + \text{RBP} \underset{K_D}{\rightleftharpoons} \text{FMN} \cdot \text{RBP} \quad (2)$$

where K_D is the dissociation constant. Each data point in Figure 5 can be used to estimate K_D for the reaction 2, since the fraction of FMN that is bound to RBP, f, is given by

$$f = 1 - (F/F_0)$$

where F_0 is the original fluorescence intensity of FMN and F is the remaining fluorescence intensity in the presence of RBP. The average K_D value, calculated from the seven data points, is $2.45 \pm 0.23 \times 10^{-6}$ M. Becvar (1973) reports for this complex $K_D = 1.37 \times 10^{-6}$ M.

In the presence of 10–30% methanol, binding of FMN to RBP can also be detected. The decrease in the strength of binding is shown in Table I, which lists the values of the average K_D for the binding of FMN to RBP at 25 °C in the presence of methanol. On the basis of enzymatic activity, it has been suggested that some enzymes, such as glutamic dehydrogenase, alcohol dehydrogenases, and papain, retain their native structure in a 10% methanol aqueous mixture (Chen, 1975). Since RBP does not have any enzymatic activity, other criteria of changes in structure must be used. The ability of the protein to bind flavins seems to be a good choice, because the

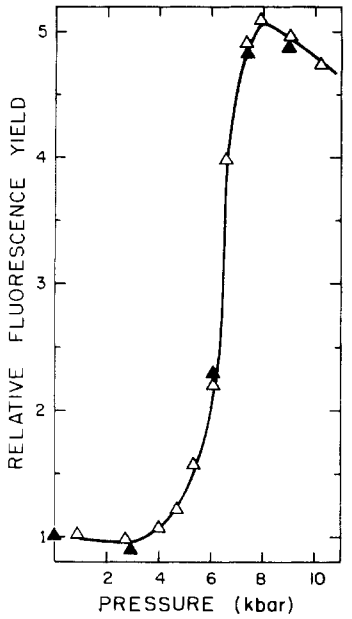

FIGURE 6: Relative ultraviolet fluorescence yield of RBP in the RBP-FMN complex vs. pressure in 0.05 M Tris-HCl buffer, pH 7.6, with 1.5×10^{-6} M RBP and 3.03×10^{-5} M FMN. Excitation wavelength was at 295 nm. At 1 atm the fluorescence efficiency of RBP in the RBP-FMN complex was ca. 0.1 times the efficiency of the apoprotein. (△) Increasing pressure, (▲) reversed.

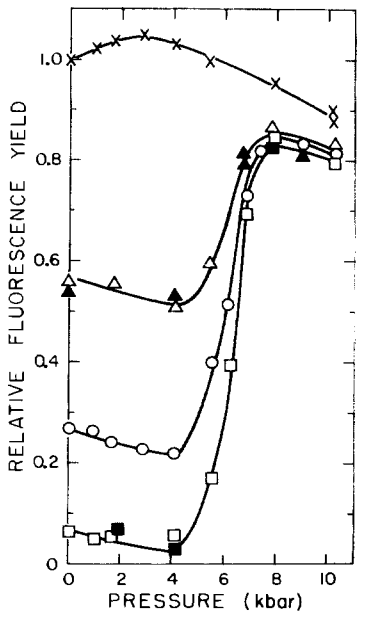

FIGURE 7: Relative fluorescence yield of FMN in the RBP-FMN complex vs. pressure. Excitation wavelength was at 375 nm. Concentration of FMN was 3×10^{-6} M: (X) FMN alone, (△) in the presence of 3 μM RBP, (○) in the presence of 9 μM RBP, (□) in the presence of 31 μM RBP. Solid symbols were data obtained when pressure was decreased after a pressure of 10 kbars was reached.

TABLE I: Effect of Methanol on the Dissociation Constant for the FMN-RBP Complex.[a]

Methanol (%)v/v	K_D
0	2.45×10^{-6} M
10	13.0×10^{-6} M
20	29.5×10^{-6} M
30	42.8×10^{-6} M

[a] At 25 °C and in 0.05 M Tris-HCl buffer, pH 7.6.

protein, when denatured by urea or guanidine hydrochloride, loses its flavin binding capacity (Rhodes et al., 1959). In addition, the absorption and circular dichroism spectra of the protein are not perturbed by 10–30% methanol, indicating the absence of significant structural changes in the proximity of the absorbing chromophores.

Pressure Effects upon FMN-RBP Complexes. The effects of pressure upon the protein's structure may be readily followed by observing the quenching of its ultraviolet fluorescence by FMN. If pressure perturbs the structure of protein, one would expect to see a variation of the amount of fluorescence quenching by FMN. Indeed, an enhancement of protein fluorescence corresponding to the dissociation of virtually all the FMN bound at atmospheric pressure is observed (Figure 6). This reversible change of fluorescence occurs in the region of 4–8 kbars, with a midpoint pressure for the transition, $P_{1/2}$, of 6.5 kbars. For a unique native to denatured transition, this midpoint pressure should correspond to the state at which native and denatured protein have equal chemical potentials, so that the conversion of one into the other is without any free energy change ($\Delta F^\circ = 0$).

The pressure dependence of the equilibrium between FMN and RBP, monitored by observing changes in the fluorescence of FMN, can provide information about the state of the binding site on the protein molecule. Figure 7 shows the pressure dependence of the FMN fluorescence when alone in solution or in the presence of various concentrations of RBP. The top curve represents the fluorescence of free FMN. It shows that there is very little variation of the fluorescence with pressure. The bottom three curves correspond to the same amount of FMN in which the fluorescence is quenched to different extent by the protein. The three curves therefore represent different degrees of saturation or different amount of complex formation. Although they start at three different levels, they all come up to the same final level. In the three cases the transition occurs within a small pressure range, with a midpoint of 6.5 kbars, and the changes are reversible.

Chemical Equilibrium of Protein and Fluorophore under Pressure. We consider only a simplified scheme of one native form, N, and one denatured form, D. Since both N and D can potentially bind the ligand X, the equilibrium reactions take the form

$$\begin{array}{ccc} NX & \overset{k_{px}}{\rightleftharpoons} & DX \\ \Big\updownarrow K_N & & \Big\updownarrow K_D \\ X + N & \underset{k_p}{\rightleftharpoons} & D + X \end{array} \qquad (3)$$

The first-order constants k_p, k_{px} are defined by the relations

$$k_p = [D]/[N]$$
$$k_{px} = [DX]/[NX] \qquad (4)$$

and the ligand dissociation constants are

$$K_N = [N][X]/[NX]$$
$$K_D = [D][X]/[DX] \qquad (5)$$

Only three of these constants are independent, since

$$K_D/K_N = K_p/k_{px} \qquad (6)$$

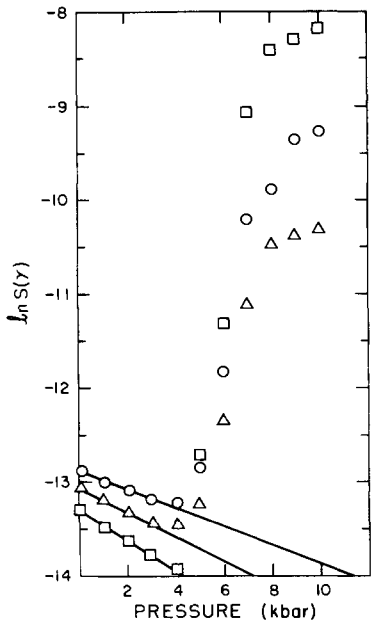

FIGURE 8: ln $S(\gamma)$ vs. pressure for RBP–FMN complex in 0.05 M Tris-HCl buffer, pH 7.6. $S(\gamma)$ is defined in the text. Symbols were defined in the legend to Figure 7.

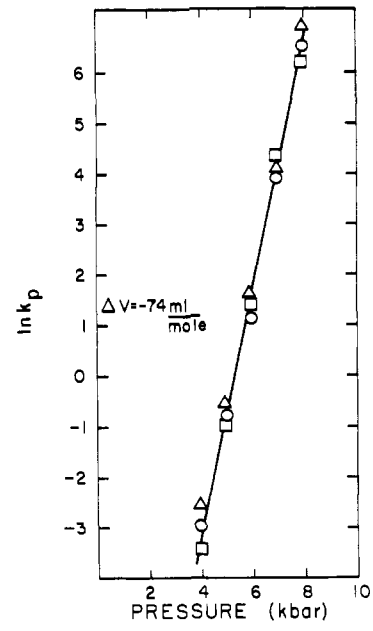

FIGURE 9: ln k_p vs. pressure for RBP–FMN complex in 0.05 M Tris-HCl buffer, pH 7.6. k_p is the equilibrium constant for the transformation of the protein from the native form to the denatured form. Symbols were defined in the legend to Figure 7.

The fluorophore X may be free, D bound, or N bound, with fluorescence efficiency q_x, q_D, or q_N, respectively. Under equilibrium conditions the fluorescence observed is

$$F = [X]q_x + [DX]q_D + [NX]q_N \quad (7)$$

The displacement of the equilibrium between N and D forms with pressure can be efficiently followed by measurements of fluorescence intensity if two conditions are fulfilled: (1) large difference between the efficiency q_x of the free form and the bound forms q_D and q_N; (2) appreciable difference in affinity for the ligand exists between the forms N and D, so that a concentration of X may be chosen at which either NX or DX will be dissociated, while the other form will not.

In the present case we have $q_N \ll q_X$. Because of the low affinity of X for the high-pressure form of the protein, q_D cannot be ascertained but in any case we can set $q_D[DX] = 0$ leaving eq 7 in the simple form

$$F = q_X[X]$$

$$\gamma = F/F_0 = [X]/X_0 \quad (8)$$

where X_0 is the total flavin in solution and F_0 the fluorescence in the absence of binding. It will be noticed that since the form DX is not detectable eq 3 reduces to

$$\begin{array}{c} NX \\ \Big\Vert K_N \\ X + N \underset{k_p}{\rightleftharpoons} D + X \end{array} \quad (9)$$

With P_0 as the total protein concentrations,

$$P_0 = [D] + [N] + [NX]$$

$$X_0 = [X] + [NX] \quad (10)$$

and using eq 8 together with eq 4 and 5

$$k_p = [D]/(P_0 - X_0(1 - \gamma) - [D])$$

$$K_N = \frac{(P_0 - X_0(1 - \gamma) - [D])\gamma}{(1 - \gamma)} \quad (11)$$

[D] may be eliminated from the above equations giving

$$S(\gamma) = [P_0 - X_0(1 - \gamma)](\gamma/(1 - \gamma)) = K_N(1 + k_p) \quad (12)$$

A plot of ln $S(\gamma)$ against pressure will yield a slowly varying value, ln K_N, in the region of low pressure, where $k_p \ll 1$. The slope in this region gives the change in volume ΔV upon dissociation of the NX complex since

$$\Delta V = \frac{-RT\mathrm{d} \ln K_N}{\mathrm{d}p}$$

In the higher pressure region where $k_p > 1$, the value of k_p and d ln K_p/dp may be calculated under the assumption that dK_N/dp = constant. Thus, both the change in volume upon formation of the protein–ligand complex and the change in volume upon denaturation may be obtained. It is to be noticed that because of the low affinity of the D form for the ligand no appreciable amounts of DX will exist, so that the values of K_D and k_{pX} are indeterminate, except for the assignment of a lower bound to K_D.

Figure 8 shows a plot of ln S vs. pressure for the FMN–RBP complex. In the pressure range of 0–4 kbars, the change in volume upon dissociation of the FMN–RBP complex is 3.3 ml/mol. In a recent study, the dissociation of the FMN–AMP complex in the pressure range of 0–4 kbars was found to proceed with a volume change equal to 4 ml/mol (Weber et al., 1974). The present study indicates that the volume change upon complex formation between FMN and RBP is not too different from that of FMN–AMP complex. The values of $S(\gamma)$ for different P_0 are quite consistent in that they yield similar values (−74 ml/mol) for the volume change from native to denatured forms for the various protein concentrations (Figure 9).

The hypothesis that globular proteins in aqueous solution are stabilized by hydrophobic bonds was first suggested by Kauzmann (1959). Although in their report on the pressure dependence of ribonuclease denaturation, Brandts et al. (1970) concluded that the currently held concept of hydrophobic bonding failed to account entirely for the denaturation changes

TABLE II: Effect of Methanol on Transition Midpoint and Volume Change upon Denaturation.[a]

% of Methanol (v/v) in Methanol: Tris-HCl Buffer Mixture	$P_{1/2}$ (kbar)	ΔV (ml/mol)	$-P_{1/2}\Delta V$ (kcal/mol)
0	6.4	−74	11.6
20	5.5	−66.5	8.9
25	4.9	−60	7.2
30	4.7	−55.6	6.4

[a] Measurements were done at 23 °C in a 0.05 M Tris-HCl buffer, pH 7.6.

roduced by pressure, it appeared of interest to study the effect f organic solvents, like methanol, on the transition midpoint pressure, $P_{1/2}$, and volume change upon denaturation of RBP. Table II shows the dependence of these quantities on the amount of methanol employed. There is a striking decrease of the transition midpoint as the percentage of methanol is increased from 0 to 30%. This shifting of the midpoint pressure is accompanied by a progressive decrease in the volume change upon denaturation.

At constant temperature the following simple transition equation that relates the free energy change for denaturation, $\Delta F°$ (1 atm), to the product of $P_{1/2}$ and ΔV can easily be derived:

$$\Delta F° \ (P_{1/2}) - \Delta F° \ (1 \text{ atm}) = \Delta V P_{1/2}$$

Since $\Delta F° \ (P_{1/2})$ equals zero at $P_{1/2}$, the free energy change for denaturation can in fact be estimated by $-\Delta V P_{1/2}$. Table II shows that the free energy change for denaturation, $\Delta F°$ (1 atm), in water equals to 11.6 kcal/mol and decreases progressively as the concentration of methanol in the medium increases.

Discussion

Protein denaturation, which is classically carried out either by the addition of chemical agents or by increase in temperature, has been shown in recent years to result also from the application of pressures of a few kilobars. Presently, the two fundamental questions emerging from these observations of the pressure denaturation of proteins are: (1) What is the origin of the small values of the change in volume upon denaturation, a quantity that in the cases studied has been found to be less than 0.5% of the molar volume of the protein? (2) Why does the characteristic denaturation process in the physiological pH range take place in the region of 4–8 kbars? The interpretation of the observations must always be made by reference to the method used. It is hardly necessary, but very important to remind oneself that each particular method of observation registers changes of a preferred component of the protein, and is not equally affected by the change or lack of it, of other components. Thus, in absorbancy measurements the preferred component is the set of aromatic amino acid residues, weighted according to their participation in the absorbancy changes, or the heme in a heme protein like metmyoglobin. In observations of the native fluorescence of proteins the preferred component is the set of tryptophan residues weighted according to their contribution to the fluorescence emission. In the measurement of the fluorescence of FMN in the presence of RBP one follows the properties of the flavin binding site. In this latter case the value of ΔV measured represents an average difference between the solvated volume of the native forms of the protein and of the form or forms of lesser binding affinity appearing at the higher pressure. It is by no means certain that ΔV represents the total difference in volume between the solvated protein in the native state and the forms existing in solution at a pressure of 10 kbars. Similar limitations apply to the values of ΔV previously derived in other systems from absorption spectroscopy measurements, although the similarity of the values observed by the different methods would be most simply explained if ΔV is grossly representative of volume changes over the whole protein. A closely allied question is the existence of a virtually unique denaturation transition, as opposed to a progressive unfolding with appearance of successively more denatured forms. The very small changes in absorbance and the relatively narrow range of pressure over which it takes place would certainly preclude the observation of intermediate steps in the process. Even in the observation of changes in FMN binding, in which the fluorescence intensity increases 30-fold over a small pressure range, the chances of observing intermediate steps are not good. The question as to the multiplicity of denatured forms is best answered by a comparison of the pressure effects when various methods of observation are used in the same protein, and by a study of the properties of the denatured form itself. For the present, because of the relative insensitivity of the techniques and the narrow range of pressures over which the effects are observed, we are compelled to treat the problem as one of equilibrium between two significant forms: native and denatured. The volume changes observed in the process result from the addition of individual contributions of the amino acid residues. Based on solvent transfer data of model components, Kauzmann (1959) suggested that the unfolding of the protein aliphatic residues is accompanied by a decrease in volume. On the other hand, Kasarda (1970) by dilatometric measurements and Weber et al. (1974) by observations of chemical equilibria under pressure have shown that unfolding of aromatic rings is accompanied by increase in volume, although the "hydrophobic" nature of the interactions is believed to be the same for the two kinds of residues, aliphatic and aromatic. Thus, the simplest explanation of the observed small value of ΔV is that it results from incomplete compensation of opposing effects owing to aliphatic and aromatic residues. The effects of the high effective concentration of residues, pointed out by Boje and Hvidt (1972), preclude a simple computation of the effects from those observed in dilute, thermodynamically ideal systems, and likewise may be responsible for the small values observed.

There appears to be no simple explanation at present for the relatively narrow range of $P_{1/2}$ values observed. It is interesting in this respect to consider a further driving force that should lead to the appearance of an unfolded form at high pressure that has not been explicitly considered up to now: the specific folding of a peptide chain into a globular structure not penetrated by solvent is achieved through a rigorous fitting of the volume of the amino acid residues attached at regular intervals along the backbone of the peptide chain, to the demands imposed by bond distances and angles of the backbone. These must be considered virtually unchangeable over the range of 10 kbars. Owing to this property the "interior" of a globular protein may be considered as relatively incompressible, leaving the amino acid residues on the surface of contact with the solvent as the ones that undergo the main decrease in volume when the pressure is applied. Evidently, an increase in the surface of contact with the solvent, as takes place upon unfolding, must lead to a decrease in volume of the system. At present we are not able to make a detailed computation that would permit us to assess the importance of this driving force

as opposed to others but the fact that it provides a homogeneous cause present throughout the whole globular protein and that it should be present to similar extent in all globular proteins could well explain the narrow range of $P_{1/2}$ values.

Conclusions

Of the observations reported here there are four that we consider important in facilitating the study and in adding some detail of interest to our knowledge of the effects of high pressure upon proteins.

First, the observation of the reversible displacement of the tryptophan fluorescence maximum from 342 nm, characteristic of the native protein, to the high-pressure value of 354 nm, indistinguishable from that of tryptophan in water, provides independent evidence that in going to the high-pressure form the surroundings of these amino acid residues are replaced by water to considerable extent.

Second, we have shown that in the study of the equilibrium of a small specific ligand with the protein it is possible to separately estimate the change in volume upon formation of the ligand–protein complex and distinguish it from a much larger volume change that is assigned to the transformation of the native to the denatured form.

Third, we have demonstrated that upon addition of an organic solvent, methanol, the denaturation process is facilitated, as revealed by a decrease in both $P_{1/2}$ and ΔV. This observation is in agreement with the general concept of the stabilization of the globular structure of the protein by hydrophobic bonds (Nemethy and Scheraga, 1962).

Fourth, the measurements convincingly demonstrate the sensitivity that can be achieved in determining the parameters of the transformation N $\rightarrow$ D by a study of the fluorescence, either of the protein or of a specifically bound ligand. In the latter case the concentrations of the protein and ligand at equilibrium could be adjusted to yield a 30-fold change in the signal when the pressure is increased, a change of a different order of magnitude from those observed up to now in these studies.

In the present case, observations of fluorescence lifetime and polarization have not been made. Such observations should provide in the future a more complete picture of the pressure effects upon both the protein and the protein complex with fluorescent ligands.

References

Becvar, J. E. (1973), Ph.D. Thesis, University of Michigan, Ann Arbor, Mich.

Boje, L., and Hvidt, A. (1972), *Biopolymers 11,* 2357.

Brandts, J. F., Oliveira, R. J., and Westort, C. (1970), *Biochemistry 9,* 1038.

Chen, R. F. (1975), *Arch. Biochem. Biophys. 166,* 584.

Drotning, W. D. (1975), Ph.D. Thesis, University of Illinois, Urbana, Ill.

Hawley, S. A. (1971), *Biochemistry 10,* 2436.

Hawley, S. A., and Mitchell, R. M. (1975), *Biochemistry 14,* 3257.

Kasarda, D. D. (1970), *Biochim. Biophys. Acta 217,* 535.

Kauzmann, W. (1959), *Adv. Protein Chem. 14,* 1.

Massey, V., and Swoboda, B. E. P. (1963), *Biochem. Z. 338,* 474.

Nemethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66,* 1773.

Neuman, R. C., Kauzmann, W., and Zipp, A. (1973), *J. Phys. Chem. 77,* 2687.

Okamoto, B. Y. (1974), Ph.D. Thesis, University of Illinois, Urbana, Ill.

Rhodes, M. B., Bennett, N., and Feeney, R. E. (1959), *J. Biol. Chem. 234,* 2054.

Weber, G. (1950), *Biochem. J. 47,* 114.

Weber, G. (1966), in Flavins and Flavoproteins, Slater, E. C., Ed., Amsterdam, Elsevier, p 15.

Weber, G., Tanaka, F., Okamoto, B. Y., and Drickamer, H. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1264.

Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239,* 1415.

Witkop, B. (1961), *Adv. Protein Chem. 16,* 221.

Zipp, A., and Kauzmann, W. (1973), *Biochemistry 12,* 4217.