$$T_{2N} = (2N)! \sum_{k=0}^{N} \frac{(2k-1)!!}{(2k)!} \sum_{m=0}^{2N-2k} \frac{1}{m![(2N-2k-m)!]} \quad \text{(A-6)}$$

For RNase A, with $2N = 8$, $T_{2N} = 7193$, i.e., 7191 intermediates plus the native and fully reduced species. We are indebted to Y. Isogai for this derivation and to R. A. Fredrickson for a similar one.

References

Ahmed, A. K., Schaffer, S. W., & Wetlaufer, D. B. (1975) *J. Biol. Chem. 250*, 8477.

Chavez, L. G., Jr., & Scheraga, H. A. (1980a) *Biochemistry 19*, 996.

Chavez, L. G., Jr., & Scheraga, H. A. (1980b) *Biochemistry 19*, 1005.

Creighton, T. E. (1975) *J. Mol. Biol. 95*, 167.

Creighton, T. E. (1977) *J. Mol. Biol. 113*, 329.

Creighton, T. E. (1979) *J. Mol. Biol. 129*, 411.

Davidson, B. E., & Hird, F. J. R. (1967) *Biochem. J. 104*, 480.

Dennis, J. E., & Mei, H. H. W. (1975) *An Unconstrained Optimization Algorithm which Uses Function and Gradient Values*, Technical Report No. TR75-246, Department of Computer Science, Cornell University, Ithaca, NY.

Eager, J. E., & Savige, W. E. (1963) *Photochem. Photobiol. 2*, 25.

Eisenberg, H. (1976) *Biological Macromolecules and Polyelectrolytes in Solution*, Chapter 5, Clarendon Press, Oxford.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.

Goto, Y., & Hamaguchi, K. (1979) *J. Biochem. (Tokyo) 86*, 1433.

Hantgan, R. R., Hammes, G. G., & Scheraga, H. A. (1974) *Biochemistry 13*, 3421.

Harrington, W. F., & Schellman, J. A. (1956) *C. R. Trav. Lab. Carlsberg, Ser. Chim. 30*, 21.

Hermans, J., Jr., & Scheraga, H. A. (1961) *J. Am. Chem. Soc. 83*, 3293.

Konishi, Y., & Scheraga, H. A. (1980a) *Biochemistry 19*, 1308.

Konishi, Y., & Scheraga, H. A. (1980b) *Biochemistry 19*, 1316.

Pirie, N. W., & Pinhey, K. G. (1929) *J. Biol. Chem. 84*, 321.

Saxena, V. P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015.

Schaffer, S. W., Ahmed, A. K., & Wetlaufer, D. B. (1975) *J. Biol. Chem. 250*, 8483.

Scheraga, H. A. (1980) in *Protein Folding* (Jaenicke, R., Ed.) p 261, Elsevier, Amsterdam.

Schöberl, A., & Gräfje, H. (1958) *Justus Liebigs Ann. Chem. 617*, 71.

Sela, M., & Lifson, S. (1959) *Biochim. Biophys. Acta 36*, 471.

Smyth, D. G., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 227.

Spackman, D. H., Stein, W. H., & Moore, S. (1960) *J. Biol. Chem. 235*, 648.

Taniuchi, H. (1970) *J. Biol. Chem. 245*, 5459.

Effect of Hydrostatic Pressure on Lysozyme and Chymotrypsinogen Detected by Fluorescence Polarization†

G. S. Chryssomallis, P. M. Torgerson,‡ H. G. Drickamer, and G. Weber*

ABSTRACT: The effect of hydrostatic pressure upon solutions of chymotrypsinogen and lysozyme at room temperature has been followed by employing a new technique [Chryssomallis, G. S., Drickamer, H. G., & Weber, G. (1978) *J. Appl. Phys. 49*, 3084] that permits the measurement of fluorescence polarization at pressures of up to 10 kbar. Lysozyme shows a stable, reversible 60% increase in apparent volume when the pressure is raised to 9 kbar. This can be given a simple interpretation in terms of solvent penetration of the structure at higher pressures. In contrast, the results with chymotrypsinogen are time dependent and only partially reversible on release of the pressure. They involve conversion ($t_{1/e} = 5$ min) to a form with a lower rotational rate at approximately 6 kbar and return to a fast-rotating form at higher pressure. This latter form persists on pressure release. The possibility of generating what are clearly metastable conformations, not only in chymotrypsinogen but also in flavodoxins [Visser, A. J. W. G., Li, T. M., Drickamer, H. G., & Weber, G. (1977) *Biochemistry 16*, 4879], indicates that there are unresolved questions about the relative stability of protein conformations which can be profitably investigated by high-pressure experiments.

The perturbation of protein structure due to hydrostatic pressure has been under investigation for several years by various methods, including absorption spectroscopy (Brandts et al., 1970; Hawley, 1971; Zipp & Kauzmann, 1973), electrophoresis (Hawley & Mitchell, 1975), and fluorescence spectroscopy, either of the intrinsic tryptophans or of added ligands (Heremans et al., 1974; Li et al., 1976a,b; Visser et al., 1977). These investigations have demonstrated that pressure can alter the affinity of proteins for small molecules, either increasing or decreasing it, depending on the nature of the binding site (Togerson et al., 1979). They also have shown that pressure perturbs the environment of tryptophan, increasing its exposure to the solvent as compared to the native protein in the case of lysozyme (Li et al., 1976b). All these data point to the appearance of large changes in the protein structure at the higher pressures, and by analogy with the denaturation reactions (Tanford, 1968), one must expect considerable changes in the hydrodynamic properties. No data on this point have been recorded, but the recent development

† From the Department of Biochemistry (P.M.T. and G.W.) and the School of Chemical Sciences and Materials Research Laboratory (G.S.C. and H.G.D.), University of Illinois, Urbana, Illinois 61801. *Received December 15, 1980.* This work was supported in part by Grant 11223 of the National Institute of General Medical Sciences, U.S. Public Health Service, and in part by the U.S. Department of Energy under Contract DE-AC02-76-ER01198.

‡ Present address: Cardiovascular Research Institute, University of California, San Francisco, CA 94143.

0006-2960/81/0420-3955$01.25/0 © 1981 American Chemical Society

of an instrument capable of measuring fluorescence polarization at pressures up to 11 kbar (Chryssomallis et al., 1978) has made the study of rotational motions of the protein possible, providing another source of information about its structure. Knowledge of the polarization, coupled with the fluorescence lifetime, allows calculation of the Debye rotational relaxation time, itself dependent upon the size and shape of the rotating element (Weber, 1953). The two proteins chosen for this study are hen egg white lysozyme and bovine chymotrypsinogen A, for which data as regards their fluorescence properties under pressure have been recorded (Li et al., 1976b). Observations on the polarization of the intrinsic fluorescence of the proteins as well as that of fluorescent conjugates of suitable long lifetime are possible. The value of the former is limited by the heterogeneous character of the emission arising from the presence of several different tryptophans, by the possibility of changes with pressure of the complex excitation–polarization spectrum (Weber, 1960; Valeur & Weber, 1977), and by the short fluorescent lifetime which gives preponderant weight to local fast rotations of the residues (Munro et al., 1979; Lakowicz & Weber, 1980) and lesser weight to the slow overall protein rotations. Fluorescein conjugates of both proteins are therefore used, since this probe has a high quantum yield and a lifetime which is near optimum for proteins in this size range.

Materials and Methods

Lysozyme and chymotrypsinogen A were products of Worthington Biochemical Corp. Their concentrations were determined by using absorbances at 280 nm of 26.5 and 20.4, respectively, for the 1% solutions. Fluorescein isothiocyanate (isomer I) was a product of Sigma Chemical Co. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, used since it exhibits only a slight dependence of its pK_a upon pressure (Neuman et al., 1973), was prepared from Trizma base (Sigma).

Fluorescein labeling of proteins was carried out by adding ca. 0.1 mg of fluorescein isothiocyanate in 0.05 M Tris–acetate buffer, pH 8.0, to 5 mg of protein in 1 mL of the same buffer. Free uncoupled fluorophor was eliminated by chromatography on Sephadex G-25, followed by dialysis against 0.05 M Tris–acetate buffer, pH 8.0. The concentration of bound fluorescein was determined by using a molar extinction coefficient of 42 500 at 490 nm (Gennis et al., 1972). Labeling ratios in all cases were less than 0.2 fluorophor per protein. For all experiments involving lysozyme, the protein concentration was less than 10^{-5} M, and for those involving chymotrypsinogen, it was less than 2.5×10^{-5} M.

Polarization values at 1 atmosphere were obtained with the polarization photometer described by Jameson et al. (1978). Isothermal Perrin plots at atmospheric pressure were constructed by varying the viscosity by addition of sucrose. Polarizations under pressure were measured with the instrument of Chryssomallis et al. (1978). Since this polarization photometer measures relative rather than absolute polarization, all pressure measurements used the known atmospheric value as the starting point. Emission spectra at high pressures were recorded with a photon-counting emission spectrometer (Li et al., 1976a). Fluorescence lifetimes were measured with single photon counting techniques. The apparatus (Klick et al., 1977) was modified by using a nitrogen-filled light source (Photochemical Research Associates, Inc., Model 510) which produces a light pulse of approximately 3-ns width. Consequently, lifetimes in the range of 3–4 ns require a deconvolution routine. The decay of the fluorescence is detected by an EMI9813QB photomultiplier tube.

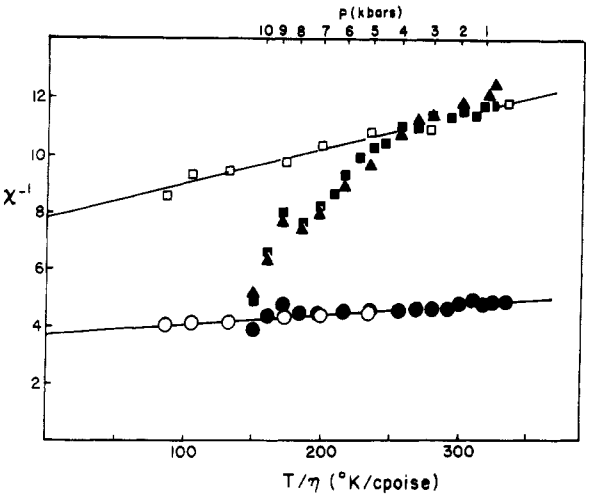

FIGURE 1: Perrin plots of lysozyme from intrinsic tryptophan fluorescence: (□) 1 atmosphere results, excitation at 286 nm; (■, ▲) pressure results, excitation at 286 nm; (○) 1 atmosphere results, excitation at 300 nm; (●) pressure results, excitation at 300 nm.

Results

Intrinsic Lysozyme Fluorescence. The effect of pressure on the intrinsic fluorescence polarization of lysozyme is shown in Figure 1, as a Perrin plot. Expressing the results in this way facilitates distinction of polarization changes caused by the pressure-induced alterations in the protein structure from those caused by the pressure-mediated solvent viscosity increase. The results of an experiment at atmospheric pressure are also shown for comparison. At an excitation wavelength of 286 nm, the polarizaiton under pressure began to deviate from the values at 1 atmosphere between 4 and 5 kbar, in agreement with the first denaturation step observed by Li et al. (1976b). The exact cause of this polarizaiton increase cannot be assigned, since lysozyme contains six tryptophan residues and their relative contribution to the fluorescence intensity may be changing with pressure, and in addition, the polarization spectrum of lysozyme changes very rapidly with wavelength near 286 nm (Weber, 1960). Pressure-induced changes in the polarization spectrum cannot be separated from extrinsic effects. At an excitation wavelength of 300 nm, however, the polarization of tryptophan is nearly constant with wavelength. As shown in Figure 1, at this wavelength there is little deviation between values at 1 atmosphere and under pressure until about 8 kbar. This is the pressure region in which a second denaturation step was observed by Li et al. (1976b) by intrinsic intensity and spectral shift measurements.

Pressure Response of Fluorescein-Labeled Lysozyme. The problems associated with intrinsic polarization changes can be avoided by using a fluorescence probe with a constant polarization spectrum. In addition, the labeling ratio must be kept low enough so that very few protein molecules have more than one probe attached. The pressure effect on fluorescein-labeled lysozyme is shown in Figure 2. The 1-atmosphere and high-pressure values began to diverge at about 6 kbar. The polarization increase can result from changes in the rotational relaxation time or from shortening in the fluorescence lifetime. For determination of the former from polarization measurements, the latter is required. The lifetimes are shown in Figure 3, along with the emission intensity of the fluorescein-labeled lysozyme as a function of pressure. Using a modification of the Perrin relation (Weber, 1953)

$$1/\chi - 1/3 = (1/\chi_0 - 1/3)(1 + 3\tau/\rho_h) \qquad (1)$$

where χ is the observed polarization, χ_0 is the limiting po-

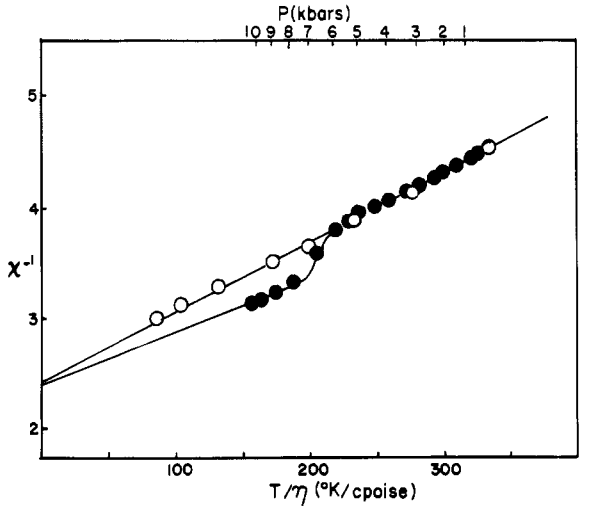

FIGURE 2: Perrin plot of fluorescein-labeled lysozyme at 1 atmosphere (○) and under pressure (●). Excitation at 490 nm.

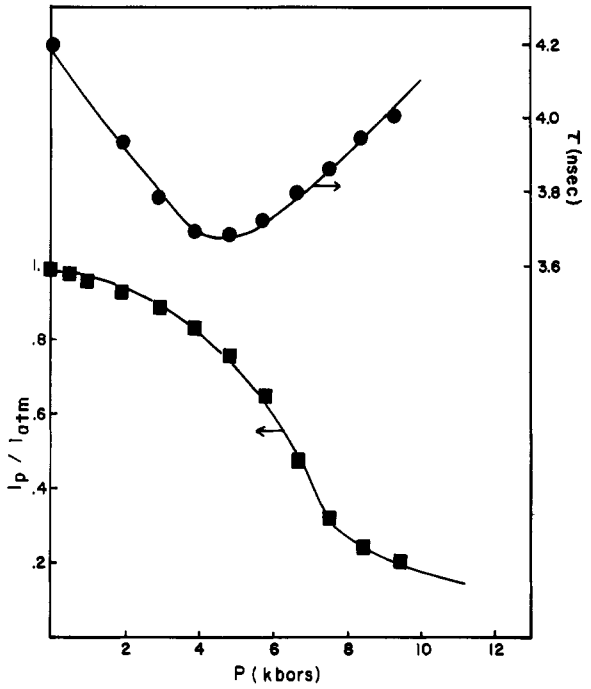

FIGURE 3: Pressure response of fluorescein-labeled lysozyme: (■) fluorescence intensity relative to 1 atmosphere; (●) fluorescence lifetime.

larization, and τ is the fluorescence lifetime, we calculated the value of ρ_h, the harmonic mean of the Debye rotational relaxation time around the three principle axes of the inertial ellipsoid. If the rotating body is approximated as a sphere, its volume (V) is the Stokes–Einstein volume, given by $V = RT\rho_h/3\eta$. ρ_h is then proportional to the volume of the rotating element and to the viscosity. Since the viscosity of water as a function of pressure is known (Bridgman, 1925, 1926), all rotational relaxation times can be corrected to constant viscosity, so as to uniquely reflect alterations in protein volume. These results are shown in Figure 4 along with the known compression of water (Bridgman, 1935, 1949). For the first few kilobars, the protein shows an apparent volume decrease approximately parallel to that of the surrounding solvent. Beginning at 5–6 kbar, however, one observes a large increase in the volume of the rotating element. After 10 kbar, the volume increase of over 60% is complete. The rotational relaxation times for lysozyme at several pressures calculated from eq 1 are given in Table I.

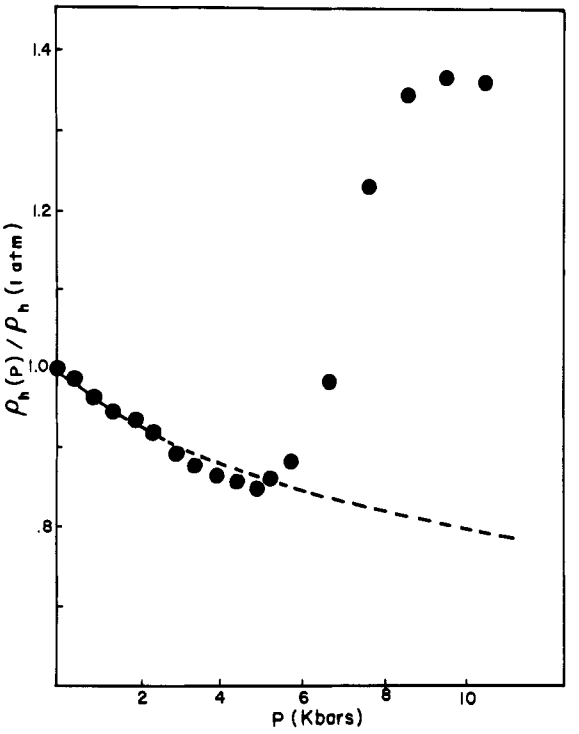

FIGURE 4: Relative rotational relaxation time of fluorescein-labeled lysozyme vs. pressure. Dashed line indicates the specific volume of water.

Table I

sample	pressure (kbar)	Debye rotational relaxation time (ns)
fluorescein-labeled lysozyme	0.001	13.3
	4.6	11.3
	9.8	18.1
fluorescein-labeled chymotrypsinogen	0.001	12.1
	6.33	28.6
	6.8	18.7
	9.8	12.2

In all measurements on lysozyme, the fluorescence parameters reached stable values within the time needed to increase pressure, usually 2–3 min. Also, the effect on lysozyme polarization was reversible. All parameters returned to their starting values upon pressure release, and a repeated pressure application gave an identical response.

Pressure Response of Fluorescein-Labeled Chymotrypsinogen. The polarization behavior of fluorescein-labeled chymotrypsinogen at 1 atmosphere and under pressure is shown in Figure 5. There is no apparent effect on the molecule up to 5.5 kbar, but at 6.33 kbar, there is a large increase in polarization. In contrast, to the case with lysozyme, however, a stable value of polarization is not reached immediately but exponentially approaches a stable value with a time constant of about 5 min.

In this figure, the point designated A was measured at 6.33 kbar immediately after raising the pressure, a procedure which requires about 2 min. The point marked B was obtained after holding this pressure for 0.5 h. The same time dependence is seen in the intrinsic tryptophan fluorescence of the unlabeled protein (inset, Figure 5). The effect is therefore not a consequence of the labeling. When the pressure is raised to 6.8 kbar, the polarization decreases with the same time constant. Again, the point marked C was measured immediately upon reaching the designated pressure, and that marked D was obtained after 0.5 h. Above this pressure, stable polarization

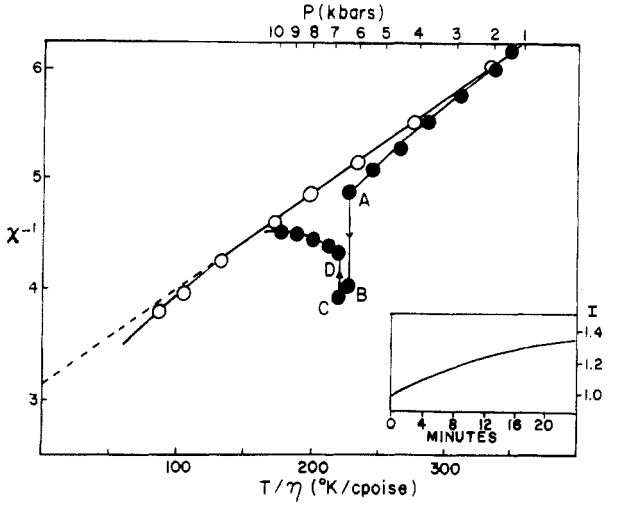

FIGURE 5: Perrin plots of fluorescein-labeled chymotrypsinogen: (○) 1 atmosphere results; (●) pressure results. Arrows indicate slow drift from initial to final values at 6.33 and 6.8 kbar. Inset: Intrinsic tryptophan emission intensity vs. time at 6.4 kbar for 2.4×10^{-6} M chymotrypsinogen.

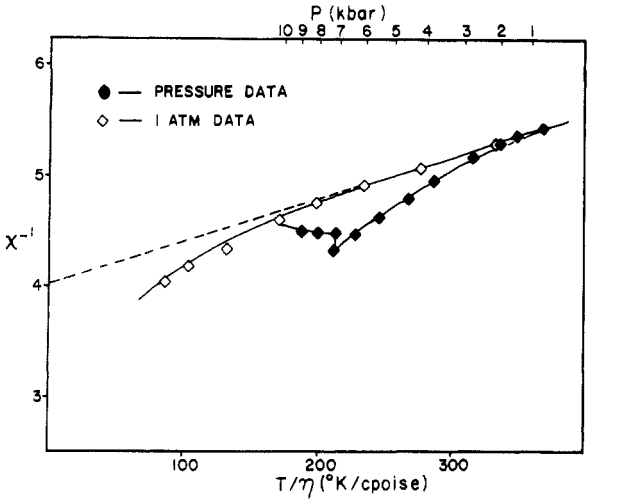

FIGURE 6: Perrin plot of pressure-denatured fluorescein-labeled chymotrypsinogen: (◇) 1 atmosphere results; (◆) results upon reraising pressure.

values are reached immediately, and there is a further slight polarization decrease. At the same pressure where polarization begins to deviate from the behavior of the native protein, the ratio of scattered light monitored at the exciting wavelength, 490 nm, to fluorescence also begins to increase. This ratio has increased by a factor of 10 at 7.5 kbar and still further above this pressure. Rotational relaxation times for fluorescein-labeled chymotrypsinogen are also given in Table I.

Also in contrast to lysozyme, the pressure denaturation of fluorescein-labeled chymotrypsinogen is not fully reversible. The scattering of the exciting light decreases markedly on decompression, but at 1 atmosphere, it is still greater than the initial scattering. However, no obvious precipitation or loss of material was observed. The 1 atmosphere and pressure polarization results for a sample previously subjected to 10 kbar are given in Figure 6. The pressure-response and 1-atmosphere behavior are both different from those of native chymotrypsinogen. The rotational relaxation time from Figure 6 is larger by factor of 2.3 than that of the native protein, and the atmospheric behavior is no longer linear. In addition, all measurements are now immediately stable, in contrast to the slowly stabilizing values observed for the native protein in the critical region of 6 kbar. The rotational relaxation time under

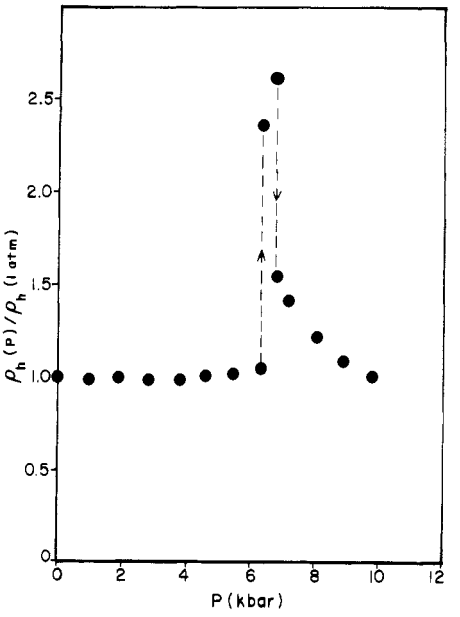

FIGURE 7: Relative rotational relaxation time of fluorescein-labeled chymotrypsinogen vs. pressure.

pressure for fluorescein-labeled chymotrypsinogen (Figure 7) illustrates the differences from lysozyme. First, the rotational relaxation time is nearly constant over the first few kilobars. Second, there is no smooth transition over a several kilobar range. Rather, the relaxation time shows a transient behavior over a narrow pressure region.

Discussion

The results with lysozyme are unequivocal in showing a large reversible increase of the order of 60% in the apparent hydrodynamic volume between 6 and 10 kbar. The two possible causes of this effect are either an actual increase in the hydrodynamic volume of the protein or a restriction in the local probe motion on the protein surface due to denaturation. Swelling of the protein due to water penetration is consistent with the observation by Li et al. (1976b) that pressure denaturation of lysozyme is accompanied by a red shift in tryptophan fluorescence, indicating exposure to a medium of higher dielectric constant. Alternatively, lysozyme is a protein consisting of two lobes, one on either side of the cleft which defines the active site. Movement of these two lobes, making the molecule more prolate, could also contribute to the observed relaxation time. However, in globular proteins, an increase in the apparent volume of over a factor of two has virtually never been observed to arise from simple changes in the frictional coefficient or from the freezing of the local rotations of the attached label, and it is therefore reasonable to assume that the larger part results from a real increase in volume of the rotating unit. This can be easily rationalized in terms of infiltration of the structure by water at the higher pressures. This penetration of solvent into the structure would in turn result in the decrease or disappearance of packing defects in the protein (Richards, 1979), which is, in our view, the main cause of the negative ΔV^0 observed in this reaction (Li et al., 1976b). At the same time, the observations show quite clearly that the increased contact of protein and solvent does not lead to the appearance of important modes of local rotation. These would result in a decrease in ρ_h below its value at atmospheric pressure, as happens, for example, in the N–F transition in bovine serum albumin (Weber & Young, 1964). The observation also supports the existence of multiple independent pressure denaturation steps, since the larger polari-

zation changes occur in a pressure region somewhat different from either of the two denaturation steps observed by Li et al. (1976b) using intrinsic tryptophan fluorescence.

The results with chymotrypsinogen are more difficult to interpret. The large increase in scattered light simultaneous with the deviation of the polarization from that of the native protein indicates that aggregation is occurring. This is supported by the increased rotational relaxation time measured at 1 atmosphere for previously pressurized chymotrypsinogen. The curvature apparent in the 1-atmosphere plot is indicative of the presence of more than one rotational relaxation time. Ordinarily, this curvature exists, one relaxation time being that of the protein and the other that of local motion of the attached probe. For that situation, however, curvature is only evident at much larger viscosities, that is, closer to the T/η origin than those employed in our experiments. Curvature at the lower viscosities, as seen in Figure 6, is indicative of a population of relatively similar rotational relaxation times. It is therefore not possible to separate the change in rotational relaxation time caused by a change in conformation of the single-chain protein from those caused by aggregation. Such self-association has been previously observed for chymotrypsinogen at 1 atmosphere (Hancock & Williams, 1969; Osborne & Steiner, 1972; Tung & Steiner, 1974). However, higher protein concentrations than those used here are necessary, and the aggregation is a reversible process. If pressure merely enhanced the affinity of chymotrypsinogen toward itself, the aggregates should dissociate rapidly upon returning to atmospheric pressure. This supports the conclusion that association is a consequence of the altered conformation of the protein under pressure. The present results are in contrast to those of Li et al. (1976b), who uniformly observed reversible pressure effects on chymotrypsinogen. The most likely reason for the discrepancy is that in the experiments of Li, insufficient time was allowed at high pressure for significant aggregation to occur. Irreversible effects of the pressure upon the protein conformation, that still await for an explanation, were noticed in flavodoxins by Visser et al. (1977). Our preliminary conclusion is that the conformation reached at high pressure can persist, either apparently helped by aggregation (chymotrypsinogen) or even without it (flavodoxins). Further investigation of this problem would be of value regarding an often posed but still incompletely solved question, the persistence of various protein conformations. The different behavior of lysozyme and chymotrypsinogen between 1 atmosphere and 6 kbar should be noted. Lysozyme appears highly compressible in this range, approximately equal to that of water (Figure 4). Chymotrypsinogen, on the other hand, appears incompressible (Figure 7). Measurements on the adiabatic compressibilities of several proteins, including both lysozyme and chymotrypsinogen, by Gratton (E. Gratton, unpublished experiments) and Gekko & Noguchi (1979) have yielded values at room temperature of between 10 and 20% of the value of water. The rotational relaxation time of lysozyme in the first few kilobars is decreasing faster than the bulk protein volume and must therefore be due to changes in the fluorophor rotation due to other causes. Chymotrypsinogen, however, appears as nearly incompressible, in agreement with the adiabatic compressibility data.

In conclusion, polarization measurements are capable of detecting changes in the overall rotational rate in proteins under pressure not directly detected by other spectroscopic techniques. In the case of lysozyme, the high-pressure increase in ρ_h arises from an important (60%) increase in particle volume. The transient in the order of minutes in the increase in polarization observed in the chymotrypsinogen case, both in the native fluorescence and in that of conjugates, offers evidence of the complexity of the reactions by which equilibrium is reached when the pressure is raised. As pointed out above, both inter- and intramolecular effects may be involved.

References

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

Bridgman, P. W. (1925) *Proc. Natl. Acad. Sci. U.S.A. 11*, 608.

Bridgman, P. W. (1926) *Proc. Am. Acad. Arts Sci. 61*, 57.

Bridgman, P. W. (1935) *J. Chem. Phys. 3*, 597.

Bridgman, P. W. (1949) *Proc. Am. Acad. Arts Sci. 77*, 129.

Chryssomallis, G. S., Drickamer, H. G., & Weber, G. (1978) *J. Appl. Phys. 49*, 3084.

Gekko, K., & Noguchi, H. (1979) *J. Phys. Chem. 83*, 2706.

Gennis, L. S., Gennis, R. B., & Cantor, C. R. (1972) *Biochemistry 11*, 2517.

Hancock, D. K., & Williams, J. W. (1969) *Biochemistry 8*, 2598.

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

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

Heremans, K. A. H., Snauwaert, J., Vandersypen, H. A., & Nuland, Y. V. (1974) *Proc. Int. Conf. High Pressure, 4th*, 623.

Jameson, D. M., Weber, G., Spencer, R. D., & Mitchell, G. (1978) *Rev. Sci. Instrum. 49*, 510.

Klick, D. I., Bieg, K. W., & Drickamer, H. G. (1977) *Phys. Rev. B: Solid State 16*, 4599.

Lakowicz, J. R., & Weber, G. (1980) *Biophys. J. 11*, 591.

Li, T. M., Hook, J. W., Drickamer, H. G., & Weber, G. (1976a) *Biochemistry 15*, 3205.

Li, T. M., Hook, J. W., Drickamer, H. G., & Weber, G. (1976b) *Biochemistry 15*, 5571.

Munro, I., Pecht, I., & Stryer, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 56.

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

Osborne, J., & Steiner, R. F. (1972) *Arch. Biochem. Biophys. 152*, 849.

Richards, F. M. (1979) *Carlsberg Res. Commun. 44*, 47.

Tanford, C. (1968) *Adv. Protein Chem. 23*, 121.

Torgerson, P. M., Drickamer, H. G., & Weber, G. (1979) *Biochemistry 18*, 3079.

Tung, M. S., & Steiner, R. F. (1974) *Eur. J. Biochem. 44*, 49.

Valeur, B., & Weber, G. (1977) *Photochem. Photobiol. 25*, 441.

Visser, A. J. W. G., Li, T. M., Drickamer, H. G., & Weber, G. (1977) *Biochemistry 16*, 4879.

Weber, G. (1953) *Adv. Protein Chem. 8*, 415.

Weber, G. (1960) *Biochem. J. 75*, 345.

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

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