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Pressure Denaturation of Metmyoglobin†

Adam Zipp and Walter Kauzmann*

ABSTRACT: When metmyoglobin is subjected to hydrostatic pressure its visible spectrum changes in a manner similar to that observed on denaturation by acid, heat, and urea. This process, which is generally reversible, was studied as a function of temperature and pH at pressures between 1 atm and 6000 kg/cm², temperatures between 5 and 80°, and pH values between 4 and 13. Combinations of pressure, temperature, and pH for the midpoint of the denaturation transition lead to a surface in (P, T, pH) space whose shape has been determined. The most striking feature of this surface is that at any pH from 4 to 13 a range of pressures exists in which the protein undergoes the changes, denatured state → native state → denatured state, when the protein is heated from 0 to 80° at constant pressure. Volume changes for denaturation were obtained by studying the effect of pressure on the equilibrium constant at several values of the pH and temperature. They are of the order of −100 ml/mol at pH <6 and −60 ml/mol at pH 10. Values for the enthalpy and entropy changes of the process under a pressure of 2800 kg/cm² were obtained at temperatures from 5 to 60°. The heat capacity change, ΔC_p, on denaturation appears to be large and relatively insensitive to pressure up to 3800 kg/cm². These results are difficult to explain if the thermodynamics of denaturation is determined by the exposure of buried nonpolar groups to water.

It is widely believed that the native conformation of a protein molecule in aqueous solution is stabilized chiefly by hydrophobic interactions (Kauzmann, 1959; Némethy and Scheraga, 1962; Eisenberg, 1970), that is, the tendency of nonpolar side chains to cluster in the interior of the protein and away from the surrounding water. If hydrophobic interactions are indeed the principal stabilizing forces in proteins and if protein denaturation involves the exposure of a significant number of nonpolar groups to the aqueous environment, then one would expect the thermodynamic changes accompanying the denaturation of proteins to resemble those of a model process in which simple nonpolar molecules are transferred from a nonpolar environment (which should resemble the interior of a protein) to an aqueous one. Certain aspects of the thermodynamics of the denaturation of ribonuclease, chymotrypsinogen, and metmyoglobin at 1 atm do show similarities to this model process (Brandts, 1969). The model is especially successful in accounting for the behavior of the heat capacity, which is much larger for the denatured protein than for the native form. The model is, however, far from successful in accounting for the changes produced by high pressure (Brandts *et al.*, 1970; Kliman, 1969).

The observed three-dimensional structure of metmyoglobin, with its predominantly hydrophobic interior (Kendrew *et al.*, 1961), makes this protein particularly well suited to the study of hydrophobic interactions as a factor in stabilizing the native structure. For this reason it seemed desirable to investigate the effect of pressure on the pH and temperature denaturation of metmyoglobin.

Information is available on the denaturation of metmyoglobin at high and low pH (Theorell and Ehrenberg, 1951; Karodjova and Atanasov, 1964; Acampora and Hermans, 1967), at high temperature (Acampora and Hermans, 1967), and in urea (Khalifah, 1968; Schechter and Epstein, 1968) as well as in the presence of various inorganic ions (Cann, 1964; Hartzell *et al.*, 1967). The denaturation of this protein as a function of temperature and pH has been studied by both optical absorbance and optical rotation measurements. The process was found to be reversible at pH below 5 and above 10 (Acampora and Hermans, 1967). A critical analysis of these data, in conjunction with potentiometric titration data (Breslow

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and Gurd, 1962) and measurements performed with a microcalorimeter (Hermans and Rialdi, 1965), has led Hermans and Acampora (1967) to conclude that the transition of native protein to denatured protein involves only two states at 1 atm. This finding encourages us to base our analysis of the effects of pressure on myoglobin on a two-state model.

Experimental Section

Materials. Sperm whale metmyoglobin (Lot 810-2510) was obtained from Sigma Chemical Co. The lyophilized protein was dissolved in distilled water, centrifuged at 12,000 rpm for approximately 15 min, and used without further purification. Concentrations were about 3×10^{-5} M for spectroscopic studies made in the visible region and about 3×10^{-6} M for studies on the Soret band.

The buffers employed were acetate, cacodylate, Tris, and glycine. All buffers were prepared from reagent grade material and all protein solutions were 0.05 M in buffer ions, with no added salt. The pH was measured with a Radiometer, Type TTT IC pH meter.

Pressure Generation System and Optical Bomb. The pressure generation system was purchased from Harwood Engineering Co., Walpole, Mass. The pressurizing fluid was spectroquality hexane obtained from Matheson Coleman and Bell. The pressure was measured by a manganin resistance coil. The complete generation and measuring system has been described elsewhere (Kliman, 1969).

The high-pressure optical bomb was designed by Professor W. B. Daniels of the University of Delaware. It was constructed from Vascomax 300 CVM maraging steel and rated to 10 kbars. The sealing capability of the high-pressure vessel was provided by unsupported area seals. To avoid contamination of the hexane pressurizing fluid, all packing material was metal. The high-pressure windows were of uv grade sapphire and were obtained from Insaco Corporation, Quakertown, Pa. The dimensions of the vessel were such that it fit into the sample compartment of a Cary 14 recording spectrophotometer. Since the pressure vessel could be reloaded in less than 5 min, it was a simple matter to perform most of the measurements on fresh metmyoglobin samples. A more complete description of the bomb can be found elsewhere (Zipp, 1973). The protein solutions were contained in a quartz internal cell of 3.4-cm path length and 1.0 cm o.d. and were isolated from the pressure fluid by means of a flexible Teflon sleeve. This cell has been described by Kliman (1969).

Constant temperature was maintained by means of a copper–brass jacket which fit snugly over the entire length of the high-pressure vessel. The circulating fluid was water, pumped through the jacket from a thermostated bath. The relationship between the bath and sample temperatures was established by inserting a copper–constantan thermocouple into the quartz–Teflon cell location in the bomb and, with the bomb set in place in the spectrophotometer sample compartment, measuring the temperature in the bomb at a series of bath temperatures between 5 and 85°. The bath temperature was maintained constant to ±0.05°.

There are small temperature changes in hexane and (to a lesser extent) in water when the pressure is suddenly changed. Since the volume of the hexane pressure fluid inside the fully loaded bomb is small (less than 2 ml), only a short time (less than 1 min) was required for temperature reequilibration after a change in pressure. Spectral measurements could, therefore, be begun as soon as the desired pressure had been reached.

Correction for Solvent Compression. The absorbance of any solution in an optical cell of fixed length will increase with increasing pressure due to compression. Before two-state equilibrium constants can be determined, the experimentally determined absorbances must be corrected for this effect. This correction can be achieved by multiplying the experimental absorbance values by the relative volume, $V(P, T)/V(1 \text{ atm}, T)$, of the solvent. The pressure and temperature dependence of the relative volume of water was taken from Bridgman (1931) and Vedam and Holton (1968).

Spectral Measurements. Spectra were measured on a Cary 14 recording spectrophotometer. The usual procedure was to equilibrate first at 1 atm and 20°, then to raise the pressure to a value which insured that the protein would be native at the desired temperature and pH. The temperature was then raised at this pressure and the bomb was allowed to equilibrate (~15 min).

At certain temperatures and pH values the two-state equilibrium constant was determined at several pressures. Except at 60° and pH 5.5, a fresh metmyoglobin sample was used for each pressure and temperature in these measurements. In many experiments a less precise measurement of the pressure at which denaturation occurs was desired. Here the same sample was used at a series of pressures for each temperature and pH studied. Spectra were always taken before applying pressure and after the release of pressure to check the reversibility of the pressure-induced spectral change.

Effect of Pressure and Temperature on the pH of Buffers. The results to be presented in subsequent sections were obtained over a relatively wide pH region (pH 4–13). Acetate buffer was employed for the range pH 4–5.5, cacodylate for the range pH 5.5–7.0, Tris (tris(hydroxymethyl)aminomethane) for pH 7 and 8, and glycine for pH 9 and 10. At pH 11, 12, and 13, NaOH was used.

The pH of a buffer solution may increase or decrease with increasing temperature and pressure, depending on the signs of the enthalpy and volume changes for ionization of the buffer acid. Therefore, in order to interpret meaningfully the results of the present study, it is necessary to know the temperature and pressure dependence of the pH for the buffers used.[1]

[1] Consider the processes

$$N \longrightarrow D + nH^+ \quad (1)$$

$$nH^+ + nA^- \longrightarrow nHA \quad (2)$$

where HA is the buffer acid. When process (1) takes place in the presence of a buffer the overall reaction is

$$N + nA^- \longrightarrow D + nHA \quad (3)$$

If the effects of temperature and pressure on the N $\rightleftarrows$ D equilibrium constant are studied in the presence of a buffer, the values of ΔH and ΔV obtained from these effects are those for process (3) and therefore depend in part on the buffer system used. Of course the reaction of interest is process (1). Since process (3) is the sum of processes (1) and (2) it is desirable that ΔH and ΔV be small for process (2) and buffers should be selected with this in mind. If this is not possible then appropriate corrections for reaction (2) must be introduced. Note that ΔV is unusually large (+25 ml) for phosphate buffers, which should therefore be avoided in studies of the effects of pressure on thermodynamic equilibria. Furthermore, ΔH for glycine and Tris are large (~10 kcal for α-amino ionization of glycine and ~12 kcal for Tris) so that significant temperature corrections can be expected when glycine and Tris buffers are used. If no buffer is present and the pH is neither high nor low, then the charge on the protein remains constant when N is converted to D, and the pH of the solution adjusts itself accordingly. In this case the process is simply

$$N \longrightarrow D \quad (4)$$

and there are no complications due to buffer ionization. Therefore it may often be desirable to study such equilibria in the complete absence of buffers.

An optical technique has recently been developed (Neuman *et al.*, 1973) which makes it possible to determine the pH of a buffer as a function of pressure. The technique is based on the known behavior of acetate under pressure and was applied to phosphate, cacodylate, and Tris buffers. This work enables us to know with a reasonable accuracy the pH at which certain of our thermodynamic results under pressure were determined. Both the 1 atm, room temperature pH and the pH at the temperature and pressure of interest will usually be given in reporting our results.

The optical technique has not been applied to the α-amino ionization of glycine. Since, however, the volume change accompanying this ionization of glycine in water is small (so that the pH change with pressure will be small, Kauzmann *et al.*, 1962), we feel it is reasonable to assume that the pH changes of glycine buffer under pressure will behave like those of Tris. The pH values of NaOH solutions at pH 11–13 will not depend on the pressure because of the large excess of OH^- ions present.

Since the temperature is also varied over a fairly wide range in these experiments, the temperature dependence of the pH of our buffers must be considered. This could be an important factor in the interpretation of experiments in which glycine and Tris buffers were used at several temperatures. It is less important, however, for Tris buffer because precipitation at high pressure (see Results) precludes any detailed analysis of the thermodynamics. For glycine the required corrections can be made from the data of Owen (1934) and King (1951).

Results

Spectral Changes Accompanying Metmyoglobin Denaturation by Pressure. Since metmyoglobin contains a heme chromophore, it has characteristic absorption bands in the 400–700-mμ region of the spectrum. Therefore, unlike many proteins for which the transition from the native to the denatured state can be followed spectroscopically only by changes in the uv (270–290 mμ), the denaturation of metmyoglobin can be followed in the visible.

When an aqueous solution of metmyoglobin is subjected to hydrostatic pressure, its visible spectrum undergoes a significant change in shape. This change is complete over a relatively narrow pressure range, suggesting a cooperative process. Figure 1 shows a typical plot of absorbance (corrected for solvent compression) *vs.* pressure at a single wavelength. As the pressure is increased from 1 atm, the spectrum corresponds to that of the native protein until the transition region is reached. At this point, the spectrum begins to change significantly with time, eventually reaching an equilibrium value at each pressure in the transition region. At pressures above the transition region the shape of the new spectrum remains unchanged with a further increase in pressure.[2] This new spectrum is considered to be that of the pressure-denatured protein.

The spectrum of pressure-denatured metmyoglobin depends on the pH and temperature. Figure 2 shows spectra of the native and denatured protein in both the visible and the Soret regions at alkaline, intermediate, and acid pH. Only spectra corresponding to the native (1 atm) and completely denatured (high pressure) states at 20° are shown.

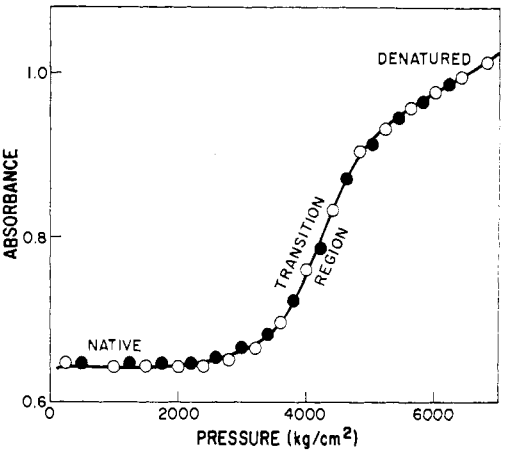

FIGURE 1: The pressure denaturation of metmyoglobin at 60°. 0.05 M acetate buffer, pH 4.9 at transition midpoint, pH 5.5 at 1 atm: (○) measurements made on increasing pressure; (●) measurements on decreasing pressure after the protein had been denatured. Absorbance measured at 536 mμ.

At pH 6.3 (Figure 2B) the visible spectrum of the native protein is characteristic of a hemoprotein with the Fe^{III} atom of the porphyrin nucleus in a high spin configuration (Smith and Williams, 1970). At alkaline pH the spectrum is characteristic of a mixture of high and low spin states (Figure 2A). In the pressure-denatured form the spectrum is characteristic of a hemoprotein with the Fe^{III} atom in a low spin configuration and this spectrum, with its maximum near 540 mμ, closely resembles that of a hemochromogen (Fe^{III} coordinated to six nitrogen-containing ligands, Drabkin and Austin, 1935). This pressure-induced high spin $\rightleftarrows$ low spin transition has been reported previously in connection with the pressurization of aqueous metmyoglobin fluoride (Zipp *et al.*, 1972) and some hemoglobin complexes (Fabry and Hunt, 1968). The transition is presumably due to the replacement of the ligand in the 6th coordination position (for metmyoglobin,

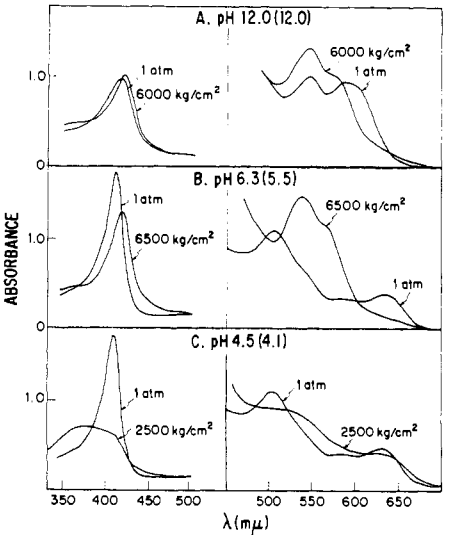

FIGURE 2: Spectral changes accompanying the pressure denaturation of metmyoglobin: (A) pH 12.0, (B) pH 6.3, and (C) pH 4.5. Numbers in parentheses indicate pH at high pressure. Protein concentration $\sim 3 \times 10^{-5}$ M in the 700–450-mμ region and $\sim 3 \times 10^{-6}$ M in the Soret region. Temperature, 20°.

[2] Under most conditions the area of the spectral band, and therefore the absorbance at any given wavelength, does not vary with pressure above the transition region. For the conditions under which the curve in Figure 1 was obtained, however, the area of the band increased with pressure above 5000 kg/cm² although the shape did not change. Therefore the absorbance continues to change with pressure above the transition region in Figure 1.

this ligand is water) by the imidazole group of a histidine residue (Zipp *et al.*, 1972).[3]

The spectral changes in the visible at 20° due to this high spin ⇄ low spin transition at alkaline and intermediate pH seem to be paralleled by the spectral changes in the Soret region (350–450 mμ). In both Figures 2A and 2B the high-pressure Soret spectrum is shifted to longer wavelengths relative to the spectrum at 1 atm. It is known that the position of the Soret absorption lies at longer wavelengths for the metmyoglobin complexes of, for example, imidazole, azide, and cyanide than for the complexes with fluoride or water; this shift is believed to reflect an increase in the amount of low spin iron present (Smith and Williams, 1970) since azide, imidazole, and cyanide (among others) are high field ligands. Therefore, it is likely that the pressure-induced wavelength shifts observed in the Soret region for metmyoglobin at alkaline and intermediate pH reflect the displacement of the high spin ⇄ low spin equilibrium to the right.

At acid pH (Figure 2C) the spectrum of pressure-denatured metmyoglobin in both the visible and the Soret regions resembles that of the acid-denatured protein at atmospheric pressure (Karodjova and Atanasov, 1964). The visible spectrum has two broad maxima, one at 60 mμ and the other at approximately 502 mμ. The high-pressure spectrum in the Soret region has decreased drastically in intensity, broadened, and shifted to shorter wavelengths compared to the 1-atm spectrum of the native protein.

Since the absorption of metmyoglobin at 400–700 mμ is due to the heme chromophore, the spectral changes observed in this region upon denaturation reflect a change in the environment of the heme and yield little direct information concerning changes in the rest of the molecule. Changes in the 270–290-mμ spectral region, on the other hand, reflect the exposure of buried aromatic side chains to the solvent. Since aromatic residues are scattered throughout the myoglobin molecule, the spectra at 270–290 mμ should yield information on the general state of folding of the protein. In order to establish whether or not changes in the folding were occurring, representative spectra at increasing pressures were taken in the 270–290-mμ region at pH 6 and 20°. They exhibited the changes typical of protein denaturation, *i.e.*, a blue shift in the absorption maximum as well as broadening of the absorption band. These changes took place in the same pressure range as the changes in the visible spectrum.

When two-state equilibrium constants for protein denaturation are obtained from changes in the absorption spectrum of colorless proteins, the uv (270–290 mμ) region must be utilized. In this region the total spectral change observed upon denaturation is typically only about 10%. Under these conditions it is more difficult to obtain accurate data since one must correct for changes in base line and, in the case of pressure experiments, for an increase in absorbance resulting from compression of the solvent. In studying the denaturation of metmyoglobin in the visible region of the spectrum, however, these difficulties have been reduced because of the comparatively large spectral changes which accompany the pressure denaturation of this protein. For example, even after appropriate correction for solvent compression and base line change, the total spectral change at pH 6.3, 536 mμ, is approximately 50% (see Figure 2B). The magnitude of this change decreases with decreasing pH, however, until it is reduced to approximately 15% at pH 4 (see Figure 2C). At these low pH values, however, the total change of the Soret absorption at 408 mμ is approximately 65%. Thus by making measurements in the visible region at the intermediate and high pH, and in the Soret region at low pH, two-state equilibrium constants could always be based on an absorbance change of at least 25%.

Since the extinction coefficient for metmyoglobin is ten times larger for absorption in the Soret region than for the 450–700-mμ region, protein solutions used for studies in the Soret region were necessarily about ten times more dilute. This approximately tenfold difference in concentration was found to have virtually no effect on the results when equilibrium constants were obtained from the two spectral regions at the same pH.

Reversibility. Two criteria were used to establish the reversibility of the pressure denaturation of metmyoglobin: (1) return of the spectrum to its 1-atm value after attainment of a time-independent value for the absorbance in the transition region at a given temperature and pressure; and (2) ability of the renatured metmyoglobin to bind oxygen upon reduction with sodium dithionite.

The return of the spectrum to its 1-atm value was tested at the end of each experiment. The absorbance change on reducing the pressure was typically 98–100% of that observed on increasing the pressure as long as the temperature was kept below 45° and no protein had precipitated at high pressure. At 60–70°, reversibility as judged by criterion (1) was still at least 95% if the pH is below 7. At pH 9–13 reversibility was less than 50% at 60° and above.

In most cases where the reversibility as judged by criterion (1) was less than 90%, the experiments had been carried out at temperatures greater than 70°, where denaturation was observed with the application of little or no pressure (~300 kg/cm²). The temperature at which denaturation occurred therefore corresponded essentially to the 1-atm value observed by Acampora and Hermans (1967), who analyzed the denaturation of metmyoglobin at 1 atm as a function of temperature and pH more carefully than we have. They concluded that even at their highest temperatures the data on the whole represented denaturation equilibria. We therefore feel that at low pressures even when the reversibility was less than 50% by criterion (1), a reversible transition was involved. In any event, if the reversibility as judged by criterion (1) is less than 95%, the data will be treated only qualitatively.

The second criterion for reversibility was applied to metmyoglobin solutions previously pressure denatured and renatured at acid, neutral, and alkaline pH at room temperature. After reversibility had been established by criterion (1), the protein was reduced with dithionite and exposed to oxygen. In all instances the resulting spectrum was that of oxymyoglobin, indicating that the renatured protein did indeed bind oxygen.

Effects of pH, Temperature, and Pressure. At a given pH and temperature, the pressure was increased in steps of 500–1000 kg/cm² until the spectrum in the 450–700-mμ region began to change. At this point, the pressure increments were reduced to 200–500 kg/cm² until successive spectra indicated that the protein was fully denatured. The midpoint of the transition was then estimated from the S-shaped curve (Figure 1) obtained by plotting pressure *vs.* absorbance at a given wavelength (536 mμ for pH 4–9, 585 mμ for pH 10–12). In order to

[3] At high temperature (>60°) and pH above 8 the visible spectrum of the denatured protein becomes broad and featureless at all pressures. An interesting exception to this is observed at pH 13 at all temperatures. If the protein is denatured either by heating at low pressure or by raising the pressure at low temperatures, the relatively featureless spectrum is obtained. If the pressure is now raised to a high value (5000–8000 kg/cm²) a new spectrum is obtained which resembles that at neutral pH, low temperature, and high pressure (Figure 2B).

TABLE I: Experimental Values of the Pressure (kg/cm²), Temperature (°C), and pH at which $\Delta G = 0$ for the Denaturation of Metmyoglobin.

Pressure	Temp	pH[a]	pH[b]	Buffer[c]
350	5	4.0	3.9	Ac
800	5	13.0	13.0	NaOH
2150	5	5.5	5.2	Ac
2800	5	6.3	5.8	Cac
2800	5	9.0	9.2	Gly
3000	5	12.0	12.0	NaOH
4250	5	10.0	10.2	Gly
600	20	4.0	3.9	Ac
1400	20	13.0	13.0	NaOH
1500	20	4.5	4.2	Ac
2500	20	5.0	4.6	Ac
3050	20	5.5	5.0	Ac
3950	20	6.3	5.7	Cac
4350	20	6.6	5.9	Cac
5000	20	12.0	12.0	NaOH
5500	20	7.0	6.2	Cac
6150	20	10.0	10.0	Gly
800	30	4.0	3.9	Ac
1000	30	13.0	13.0	NaOH
3600	40	5.0	4.5	Ac
4200	40	5.5	4.9	Ac
5075	40	6.3	5.6	Cac
		12.0	12.0	NaOH
5500	40	7.0	6.2	Cac
6000	40	10.3	10.0	Cac
6250	40	10.0	9.7	Gly
500	48	4.0	3.9	Ac
200	54	12.0	12.0	NaOH
3300	60	5.0	4.5	Ac
4150	60	5.5	4.9	Ac
4250	60	10.7	10.0	Gly
5000	60	10.0	9.3	Gly
6000	60	6.3	5.5	Cac
2500	71	5.0	4.6	Ac
4100	71	5.5	4.9	Ac
		10.0	9.0	Gly
300	74	11.0	11.0	NaOH
200	76 (±5)	5.0	5.0	Ac
		10.0	8.8	Gly

[a] As measured at 20° and 1 atm. [b] After correction for effects of temperature and pressure. [c] Ac = acetate; Cac = cacodylate; gly = Glycine.

obtain plots of pressure *vs.* temperature at constant pH, the pH of those buffers whose pH changes with pressure was varied at 1 atm to give the desired pH at high pressure. For the experimental points given, we estimate the error in the pressure corresponding to the midpoint of the transition to be ±200 kg/cm². The error in the temperature is estimated to be ±1.0°.

In correpondence with the results obtained by Acampora and Hermans (1967) at 1 atm, we find that metmyoglobin precipitates under certain conditions of pH, temperature, and pressure. The conditions for metmyoglobin precipitation are shown in Figure 3.

At 20° and pH 6 no precipitation was observed at pressures up to 9000 kg/cm², despite the fact that the spectrum indicates

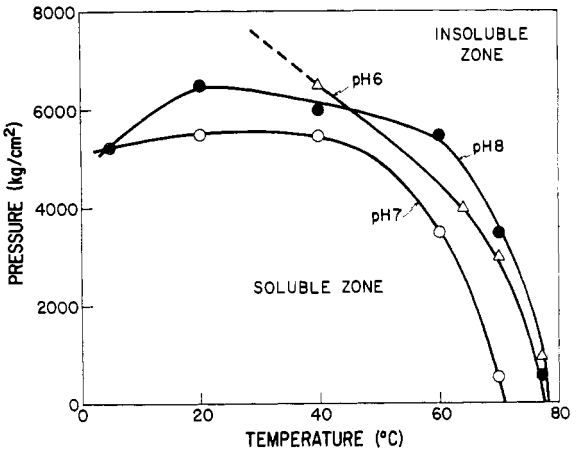

FIGURE 3: Conditions for precipitation of metmyoglobin. The pH values refer to the pH of the solution at the stated pressure at 20°.

complete denaturation at about 4500 kg/cm². It is possible that at lower temperatures the pressure tends to solubilize the denatured protein (Suzuki *et al.*, 1963a, have seen such an effect with serum albumin). Precipitation was never observed at any combination of temperature and pressure if the pH was below 5 or above 9.

The phenomenon of the pressure-induced precipitation of proteins has been observed previously (Suzuki *et al.*, 1963b). The shape of the curves at pH 7 and 8 is rather interesting. At these values of the pH, the pressure at which precipitation occurs increases approximately linearly with decreasing temperature down to about 45°, below which the slope abruptly decreases.

Table I shows the experimentally determined conditions of pH, temperature, and pressure for the midpoint of the metmyoglobin denaturation reaction (*i.e.*, conditions for which $\Delta G = 0$ for this reaction; conditions leading to precipitation are not included in this table). These data were displayed on the temperature–pressure plane and contours of constant pH were interpolated through the resulting array of points to give the curves shown in Figure 4. The most striking feature of these curves is that at any pH shown in Figure 4 a range of pressures exists in which the protein undergoes the changes, denatured state → native state → denatured state, when the protein is heated from 0 to 80° at constant pressure. For instance, metmyoglobin at pH 5, 5°, and 3000 kg/cm² will be denatured. On heating at constant pressure it undergoes a

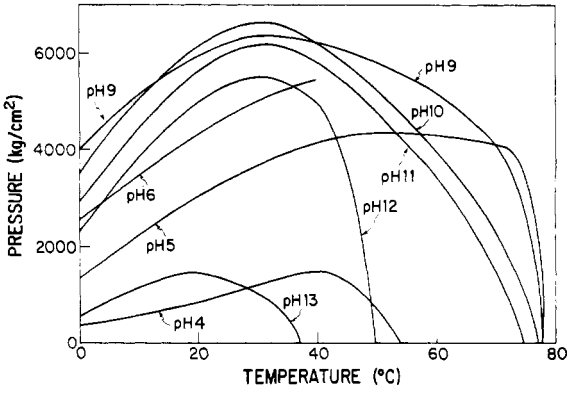

FIGURE 4: Contours of constant pH in the pressure-temperature plane at which $\Delta G = 0$ for the denaturation of metmyoglobin. The native state is more stable than the denatured state inside each contour.

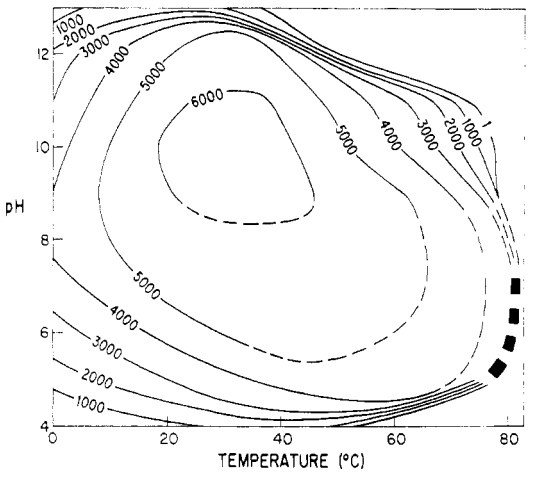

FIGURE 5: Contours of constant pressure in the pH–temperature plane at which $\Delta G = 0$ for the denaturation of metmyoglobin. The native state is more stable than the denatured state inside each contour.

transition to the native state at 20°. The temperature must now be raised to 80° in order for the protein to revert to the denatured state. The temperature of maximum stability of the native form depends upon the pH but can be remarkably high; at pH 5–6 it appears to be above 50° at 4000–6000 kg/cm^2. Similar behavior can also be observed in the results of Hawley (1971) on chymotrypsinogen at pH 2.07 where the temperature of maximum stability is about 25° at 2000–3000 kg/cm^2.

The results shown in Figure 4 can be interpreted qualitatively by the procedure used by Hawley (1971). When metmyoglobin is thermally denatured at 1 atm the entropy change, ΔS, is positive since, for a given pH, ΔG decreases with increasing temperature (Hermans and Acampora, 1967). The high-temperature end of each pH contour emerges from the abscissa ($P = 0$ axis) in Figure 4 with negative slope, so that $(dP/dT)_{\Delta G=0}$ is negative. According to the Clausius–Claperon equation

$$(dP/dT)_{\Delta G=0} = (S_D - S_N)/(V_D - V_N) = \Delta S/\Delta V$$

We therefore conclude that ΔV, the volume change for the reaction, native protein → denatured protein, must be negative at 1 atm for the entire pH range studied. Since $(dP/dT)_{\Delta G=0}$ seems to become increasingly more negative at higher temperatures, ΔS must become more positive and/or ΔV must become less negative with increasing temperature.

At each pH, $(dP/dT)_{\Delta G=0}$ becomes zero at high pressure indicating that ΔS goes to zero. As the temperature is lowered from this point, $(dP/dT)_{\Delta G=0}$ becomes positive and ΔS must become negative. Since ΔV cannot change sign in this region without making $(dP/dT)_{\Delta G=0}$ infinite (which is not observed), we conclude that ΔV must be negative at all values of the pH, temperature, and pressure that we have studied. (That ΔV is negative at low temperatures is also known from measurements described below of the effect of pressure on the equilibrium constant.) As this trend (ΔS negative, ΔV negative) continues at lower temperatures we expect a reintersection with the atmospheric pressure axis somewhere below 0°. This reintersection would correspond to the process of "cold denaturation" at 1 atm already predicted for a number of proteins (Brandts, 1969).

The constant pH contours of Figure 4 represent a three-dimensional surface in (T, P, pH) space on which $\Delta G = 0$ for the denaturation reaction. The shape of this surface is somewhat irregular and can be better visualized by plotting contours of constant pressure on the pH–temperature plane (Figure 5). These plots show that the pH of maximum stability depends in a complex way on the temperature and pressure. At 20° maximum stability toward pressure is around pH 10 whereas at higher temperatures (around 80°) the maximum stability is around pH 7. Near pH 12 and 40° the contours in Figure 5 are close together, but they appear to separate quite strongly at pH 10 and 50–80°. In addition there is some indication of an undulation of the contours in this region, but it is difficult to assess the reality of this undulation because of the poor reversibility of the denaturation reaction under these conditions.

Volume Change on Denaturation. I. Determination of ΔV from Equilibrium Constants. Assuming that the transition, native protein (N) ⇄ denatured protein (D), involves only two states, the equilibrium constant can be determined from the relation

$$K_{eq} = (A_N - A_{eq})/(A_{eq} - A_D)$$

where A_{eq} is the absorbance (corrected for compression) at equilibrium in the transition region, and A_N and A_D are the corrected absorbances for the native and denatured states under the same conditions of temperature and pressure. The values of A_N and A_D in the transition region were obtained in the usual way by linear extrapolation of data from outside the transition region.

Plots of ln K_{eq} *vs.* pressure gave straight lines within experimental error.[4] The volume changes, ΔV, for the pressure denaturation of metmyoglobin could, therefore, be calculated from the relation

$$(\partial \ln K_{eq})/\partial P = -\Delta V/RT$$

where T is the temperature in °K and R is the gas constant. Equilibrium constants were determined in the transition region on both increasing the pressure (N → D) and decreasing the pressure (D → N). In all instances volume changes were obtained from plots of ln K_{eq} *vs.* P containing at least five experimental points.

Volume Change on Denaturation. II. Correction of the Experimentally Determined ΔV for Specific Buffer Contributions. Since the volume changes obtained in this study were determined in the presence of a buffer, the contribution of the buffer ionization to the experimentally determined ΔV must be taken into account.[1] For metmyoglobin at acid pH the buffer will participate in the denaturation reaction according to the equation

$$\nu HB + N \rightleftarrows DH_\nu + \nu B^-$$

where ν is the difference in the number of protons bound by the native and denatured states and HB is the buffer conjugate acid. In order to eliminate specific effects of the buffer, ν times the volume change for the ionization of the buffer acid must be subtracted from the experimentally determined ΔV for denaturation. For the acid denaturation of metmyoglobin at 1 atm, $\nu = 6$, corresponding to the protonation of six imidazole groups which are normally buried in the native

[4] See paragraph at end of paper regarding supplementary material.

protein (Breslow and Gurd, 1962). If, for example, the denaturation equilibrium is studied in acetate buffer, a contribution of -72 ml/mol (ΔV for the ionization of acetic acid is -12 ml/mol at 1 atm, Hamann and Strauss, 1955) must be subtracted from the experimentally determined ΔV at 1 atm.

In the present studies, however, the experimentally determined ΔV's for the denaturation of metmyoglobin are not 1 atm values but were determined at various pressures and temperatures. Under these circumstances, it becomes necessary to know both ν and ΔV for the ionization of the buffer acid as a function of pressure and temperature. The pressure dependence of the volume change for the ionization of the buffer acids used here was taken from the data of Neuman *et al.* (1973). Assuming two-state behavior, ν can be determined from the relation

$$\nu = -\frac{1}{2.3}\left(\frac{\partial \ln K_{eq}}{\partial pH}\right)_{T,P}$$

Plots of ln K_{eq} *vs.* pH were obtained over a wide range of pressure[4] and from the slopes of these plots the pressure dependence of ν was obtained. The values of ν determined in this way are shown in Figure 6. Since, at constant temperature, the pressure at which the transition N $\rightleftarrows$ D takes place depends on the pH, the experimental points given in Figure 6 were necessarily determined over different pH ranges. The pH at the transition midpoint is, therefore, indicated in parentheses below each point. These pH values correspond to the pH at the pressure of interest.[5] Figure 6 shows an S-shaped curve, with ν decreasing as the pressure and pH increase. The value of ν at 1 atm reported in Figure 6 agrees with that of Breslow and Gurd (1962) within experimental error. Although the shape of the curve in Figure 6 is generally similar to the plot of ν *vs.* pH obtained by Breslow and Gurd for the denaturation of metmyoglobin at 1 atm, an important difference does exist. In the 1-atm curve ν increases more rapidly as the pH decreases near pH 5 than was found here for the same pH region at high pressure. This difference is not understood but could reflect a difference in the conformation of the protein denatured at 1 atm and at high pressure.

The first six rows of Table II give the values of ΔV for the denaturation of metmyoglobin at 20° both before and after correction for buffer participation. Although the absolute error in the corrected volume changes is probably ± 20 ml/mol because of the uncertainly in ν, the relative error should still be ± 10 ml/mol.

Values of ΔV as a function of temperature at nearly constant pH are also given in Table II. Since carboxylic acid buffers were employed, the temperature dependence of the pH can be neglected.[1] In determining the corrected volume changes at temperatures other than 20° we have assumed that ν at a given pH and pressure is independent of the temperature. We feel that this assumption is reasonable since Hermans and Acampora (1967) reported ν for the denaturation of metmyoglobin to be virtually independent of temperature from 5 to 65° at 1 atm.

Since the denaturation of metmyoglobin at acid pH involves the protonation of imidazole groups, the volume changes that we have found might be corrected even further for the contribution of this protonation. This correction would, however, be insignificant because ΔV for the protonation of imidazole is only $+1$ ml/mol and independent of the pressure. Thus the corrected volume changes in Table II at acid pH represent an inherent change in the molecule and its surroundings when it unfolds.

The volume change of about -60 ml/mol observed at pH 10 and 6000 kg/cm² probably includes a contribution from the ionization of one or two buried tyrosines (Hermans, 1962). The volume change for the ionization of a phenolic hydroxyl group is about -18 ml/mol at 1 atm (Weber, 1930; Hamann and Lim, 1954) and the pK is 10.3. At 6000 kg/cm² the pK would be at least 1 unit smaller, so that all exposed tyrosines would be completely ionized at pH 10. The volume change for the tyrosine ionization is much less negative than -18 ml/mol at 6000 kg/cm²; its actual value is not known but we estimate

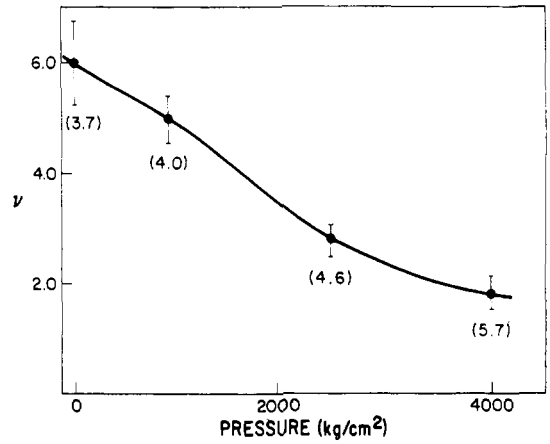

FIGURE 6: Dependence of ν on pressure for the denaturation of metmyoglobin at 20°. Buffers used are glycine–HCl at 1 atm, acetate at 1000 and 2500 kg/cm², and cacodylate at 4050 kg/cm².

TABLE II: ΔV for the Denaturation of Metmyoglobin as a Function of Pressure (kg/cm²), Temperature (°C), and pH. All Parameters Given are those at the Transition Midpoint.

Pressure	pH	Temp	ν	ΔV^a (ml/mol)	ΔV^b (ml/mol)	Buffer[c]
6000	10.0	20		−60 (±20)	−60	Gly (10.0)
4340	5.91	20	1.8	−93	−85	Cac (6.6)
3960	5.64	20	1.8	−100 (±15)	−89	Cac (6.3)
3050	5.04	20	2.3	−109	−95	Ac (5.5)
1580	4.29	20	4.3	−136	−97	Ac (4.5)
600	3.92	20	5.3	−155	−97	Ac (4.0)
2120	5.15	5	2.3	−117	−102	Ac (5.5)
3050	5.04	20	2.3	−109	−94	Ac (5.5)
4200	4.98	40	2.3	−111	−100	Ac (5.5)
4130	4.99	60	2.3	−63 (±5)	−52	Ac (5.5)
2790	5.75	5	1.8	−127	−114	Cac (6.3)
3960	5.64	20	1.8	−100	−89	Cac (6.3)
5030	5.56	40	1.8	−60	−51	Cac (6.3)

[a] ΔV = volume change before correction for buffer volume change. Estimated uncertainty is ± 10 ml/mol except where otherwise noted. [b] ΔV = volume change after correction for buffer volume change. [c] Numbers in parentheses are pH values measured at 1 atm, 25°. Cac = cacodylate; Ac = acetate.

[5] The pH at the midpoint of the denaturation transition at 1 atm obtained in these studies is somewhat lower than the value of 4.3 reported by Acampora and Hermans (1967). This discrepancy may be explained by the fact that in the present studies no salt was added to the metmyoglobin solutions whereas the Acampora–Hermans measurements were made in 0.1 N KCl.

TABLE III: Thermodynamic Parameters for the Denaturation of Metmyoglobin.[a]

Temp (°C)	ln K_{eq}	ΔG	ΔH^b	ΔS^b	$\Delta C_p{}^b$
At 2800 kg/cm², pH 5.0 (0.05 M acetate, pH 5.51 at 1 atm)					
5	3.4	−1.9 (−2.5)[c]	−84	−290	2300
20	−1.1	0.6 (0.7)	−50	−170	2300
40	−5.8	3.6 (2.6)	−4	−20	2300
60	−3.3	2.2 (1.8)	42	120	2300
At 3800 kg/cm², pH 5.7 (0.05 M cacodylate, pH 6.3 at 1 atm)					
5	5.4	−3.0	−86	−300	2700
20	−0.7	0.4	−45	−155	2700
40	−2.7	1.7	10	25	2700

[a] ΔG and ΔH in kcal, ΔS and ΔC_p in cal/deg. [b] These values calculated from eq 1 with T_0 = 273°K and, at 2800 kg/cm², ΔH_0 = −95.435, ΔS_0 = −334, ΔC_p = 2287; at 3800 kg/cm², ΔH_0 = −99,600, ΔS_0 = −348, ΔC_p = 2735. [c] Values in parentheses are calculated from eq 1 using the constants given in footnote *b*.

that it might be −5 to −10 ml/mol. Since we know neither the exact number of groups exposed nor the volume change per group at 6000 kg/cm², an accurate correction to ΔV cannot be found. The inherent volume change due to the unfolding process at pH 10 must, however, be less negative than −60 ml/mol and might well be something like −40 or −50 ml/mol.

Other Thermodynamic Parameters for the Denaturation of Metmyoglobin. In order to determine enthalpy and entropy changes on denaturation the temperature dependence of K_{eq} at constant pressure and pH must be known. The volume changes given in Table II for pH ~5 were obtained from separate plots of ln K_{eq} *vs.* P at temperatures of 5, 20, 40, and 60°. Each of these plots was extrapolated to a common pressure of 2800 kg/cm² to give the values of ln K_{eq} which are shown in Table III, along with the corresponding values of ΔG. The values of ΔH, ΔS, and ΔC_p given in Table III were obtained by a least-squares fit to these data using the relationships

$$\Delta G = \Delta H_0 + \Delta C_p(T - T_0) - T\Delta S_0 - \Delta C_p T \ln (T/T_0) \quad (1a)$$

$$\Delta H = \Delta H_0 + \Delta C_p(T - T_0) \quad (1b)$$

$$\Delta S = \Delta S_0 + \Delta C_p \ln (T/T_0) \quad (1c)$$

where ΔC_p is assumed to be independent of temperature, T_0 = 273°K, and ΔS_0 and ΔH_0 are constants. It should be noted that in order to obtain values of ln K_{eq} at 2800 kg/cm² when the temperature was 5 and 40° it was necessary to extrapolate the plots of ln K_{eq} *vs.* P quite far outside the transition region. These points are consequently somewhat less certain than the points at 20 and 60°. Table III shows a discrepancy between the extrapolated values of ΔG and those calculated assuming that ΔC_p is constant. Because of the long extrapolations at 5 and 40° this discrepancy is not considered to be particularly significant; the values of ΔH, ΔS, and ΔC_p are, in any case, to be regarded as quite approximate. These results do indicate, however, that at pH 5 and 2800 kg/cm² ΔH and ΔS are large and negative at low temperatures and become less negative with increasing temperature up to about 40°. Around 43° ΔH and ΔS change sign and ΔG passes through a maximum. This behavior of ΔH and ΔS reflects the fact that ΔC_p is large and positive. Table III also gives data obtained at 3800 kg/cm² and pH ~5.7 over a somewhat smaller temperature range. These results show a similar behavior of ΔH and ΔS, with a large positive ΔC_p. There are indications that ΔH and ΔS change sign and ΔG passes through a maximum at a somewhat lower temperature (~37°) at pH 5.7 than at pH 5.

Kinetics of Pressure Denaturation. Under certain conditions of temperature, pH, and pressure the spectral change accompanying the pressure denaturation of metmyoglobin is time dependent, eventually reaching an equilibrium value at all conditions under which the kinetics was studied. In the acid pH region, at temperatures from 5 to 40°, first-order plots for the unfolding reaction showed good linearity, suggesting two-state behavior. Half-times ranged from a few minutes for studies at pH 4.92 (4200 kg/cm², 40°) to approximately 1 hr at pH 5.79 (2800 kg/cm², 5°). The spectral change at 60° is extremely rapid at all pressures and pH values studied and is not measurable with the instrumentation used in these experiments. Some typical values of the first-order rate constants as a function of temperature, pressure, and pH are given elsewhere (Zipp, 1973).

In general, first-order plots of the refolding reaction show a biphasic character after the protein has been denatured by pressures well above the transition region. In studying the kinetics of the acid denaturation of metmyoglobin at 1 atm, Shen and Hermans (1972) found that the kinetic behavior of the refolding reaction depends on the pH conditions used to denature the protein. When the denaturing conditions were far outside the transition region, nonlinear first-order plots were found. This is consistent with our findings.

Discussion

Comparison of Denaturation by Pressure, Heat, Urea, and Acid. In the thermodynamic analysis of the results that have been presented above, we have assumed that we are dealing with a transition from native to denatured protein which involves only two species. Hermans and Acampora (1967) have presented evidence that this is a valid assumption when the temperature and pH are varied at 1 atm. The following facts indicate that the conformation change that occurs on raising the pressure at fixed pH and temperature is similar to the conformation change when the temperature is raised or when the solution is made acid at fixed pressure. (1) At a given pH and temperature the spectral changes at 700–450 mμ accompanying pressure denaturation closely resemble those obtained by thermal denaturation and by urea denaturation. At a given temperature the spectral change accompanying pressure denaturation at low pH closely resembles that produced by lowering the pH at 1 atm. These observations indicate that pressure, heat, urea, and acids produce similar structural changes in the vicinity of the heme group. (2) The spectral changes we have observed at 270–290 mμ on application of pressure are characteristic of those found for protein denaturation by other agents at 1 atm. (3) Near the isoelectric point both pressure and heat denaturation produce insoluble protein, showing that both processes expose groups (presumably nonpolar groups) of low affinity for water.

Since optical rotatory properties are strongly affected by protein conformation, it would, of course, be interesting to see if these properties of metmyoglobin are affected by pressure in the same way as by heat and acid. Unfortunately, these are not

easy to measure under pressure. It should be noted, however, that Gill and Glogovsky (1969) observed that pressure causes an increase in the levorotation of ribonuclease. Such an increase invariably accompanies the unfolding of a protein, whether induced by heat, urea, acid, or base.

Comparison of Pressure Effects on Metmyoglobin with Pressure Effects on Other Proteins and Model Systems. Protein denaturation at 1 atm is generally accompanied by large positive changes in the heat capacity, ΔC_p, presumably reflecting the exposure of nonpolar groups to the solvent water. Our results for the denaturation of metmyoglobin indicate that at 2800 and 3800 kg/cm^2, ΔC_p is still quite large and positive in accord with previous observations on metmyoglobin and other proteins at 1 atm.

There is much evidence (Brandts, 1969) that the occurrence of a temperature of maximum stability may be a general characteristic of protein denaturation. We shall define T_{max} as the temperature at which $-R \ln K_{eq}$ passes through a maximum. The recent summary by Hawley (1971) of thermodynamic studies on ribonuclease and chymotrypsinogen indicates that in the denaturation of chymotrypsinogen at 1 atm T_{max} occurs at 15° whereas for ribonuclease it occurs between 0 and −5°. From the data of Hermans and Acampora (1967) we estimate that for the denaturation of metmyoglobin at pH 5 and 1 atm T_{max} occurs near 25° (see Appendix), a value somewhat higher than that found for ribonuclease or chymotrypsinogen. (This may reflect the greater hydrophobicity of the interior of the myoglobin molecule (Brandts, 1969).) Furthermore, the data of Hawley (1971) indicate that increasing the pressure at constant pH only slightly increases T_{max} for ribonuclease and chymotrypsinogen (by about 5° for a pressure increase of 2000 kg/cm^2 for ribonuclease and 3000 kg/cm^2 for chymotrypsinogen). The data in Table III, on the other hand, suggest that T_{max} for the denaturation of metmyoglobin at pH 5 is raised from about 25° at 1 atm to about 43° at 2800 kg/cm^2, and Figure 5 shows that at 4000 kg/cm^2 T_{max} is about 50–60° at pH 5. Thus T_{max} for metmyoglobin appears to be considerably more sensitive to pressure than is T_{max} for ribonuclease and chymotrypsinogen.

In analyzing the thermodynamics of the pressure denaturation of ribonuclease and chymotrypsinogen, Hawley (1971) found a general functional form for $\Delta G(T,P)$ which for a given value of ΔG leads to elliptical contours in the pressure–temperature plane. On this basis he suggested that an ellipsoidal pressure–temperature transition surface might be a general characteristic of protein denaturation. The curves in Figure 4 for the pressure denaturation of metmyoglobin, while showing a general similarity to ellipses, are more complex in shape that those found by Hawley. Thus, although Hawley's suggestion seems to be more or less supported by our studies on metmyoglobin, it is evident that any equation which satisfactorily describes the curves in Figure 4 will be somewhat more complex than that derived for ribonuclease and chymotrypsinogen. Furthermore, even if our curves were fitted approximately to ellipses, the centers of these ellipses would move in a complex way as the pH is changed.

If protein denaturation is dominated by hydrophobic interactions then one would expect the thermodynamic changes on denaturation to parallel those that occur when nonpolar groups are transfered from a nonpolar environment (the protein interior) to water. When a small nonpolar molecule is transferred from a liquid hydrocarbon environment to water the heat capacity at 1 atm increases, and the volume at 1 atm decreases (by 10–20 ml/mol). With increasing pressure the heat capacity change, ΔC_p, decreases, becoming zero at about 5000 kg/cm^2 (Kliman, 1969); the volume change, ΔV, becomes less negative, changes sign at 1500–2000 kg/cm^2 and approaches a small positive value of around 5 ml/mol at higher pressures (Brandts, 1969; Brandts *et al.*, 1970; Kliman, 1969). Therefore, if the interior of a protein resembles a liquid hydrocarbon and if a significant number of nonpolar groups (say 10 or 20) is exposed to water on unfolding, then at 1 atm ΔC_p should be large and positive and ΔV should be large and negative (−100 to −400 ml/mol). At higher pressures ΔC_p should become smaller and ΔV should become less negative, changing sign at perhaps 2000 kg/cm^2. If the interior of the protein resembles a solid hydrocarbon, as suggested by Klapper (1971), ΔC_p would probably be even larger at 1 atm, but ΔV at 1 atm would be less negative, or even positive. Both ΔC_p and ΔV should, however, still be pressure dependent; ΔC_p would be expected to decrease with pressure, but ΔV would change sign at an even lower pressure than ~2000 kg/cm^2 and would assume considerably larger positive values at higher pressures.[6]

Hermans and Acampora (1967) have found that ΔC_p is indeed large and positive for metmyoglobin denaturation at 1 atm as expected from the above considerations. The corrected value of ΔV at low pressure given in Table II is about −100 ml/mol at 20°, which is not out of line with the expected value. There is, however, no indication of a large contribution to ΔV which becomes very much less negative or positive as the pressure is raised to 4000 kg/cm^2.[7] This is contrary to the expected behavior, regardless of whether the interior of the protein is like a liquid or like a solid. A similar observation has been made by Brandts *et al.* (1970) in their study of ribonuclease.

Furthermore, if the thermodynamic parameters of ΔS_0 and ΔC_p for the denaturation of metmyoglobin varied with pressure in the manner found by Kliman (1969), it can be shown that T_{max} should decrease with increasing pressure (see Appendix). In fact, we have seen above that the opposite is true (T_{max} for metmyoglobin at pH 5 increases from 28° at 1 atm to about 43° at 2800 kg/cm^2 and 50–60° at 4000 kg/cm^2). This predicted behavior is related to the fact that according to Kliman ΔC_p should decrease with increasing pressure. There is little indication of such a pressure dependence of ΔC_p here. The data of Acampora and Hermans (1967) seem to indicate that at pH 5, ΔC_p is about 2000 cal/(deg mol) at 1 atm and our values of ΔC_p at 2800 and 3800 kg/cm^2 do not seem to be smaller than this.

These difficulties in trying to explain the effects of pressure in terms of hydrophobic interactions are compounded by the following factor noted by Brandts *et al.* (1970). The data of Rasper and Kauzmann (1962) suggest that the molar volumes of charged amino and imidazole groups in a native protein may be larger by about 7 ml/mol than the volumes of the same groups in small molecules dissolved in water. One might therefore expect a decrease of about 7 ml/mol when a charged basic side chain in a native protein becomes "normalized" upon denaturation. Metmyoglobin contains 35 basic side chains. Therefore, when this protein is unfolded at acid pH

[6] Kuntz (1972) has estimated a density of 0.93 g cm^{-3} for the hydrophobic regions in the interior of the carboxypeptidase molecule on the basis of the observed X-ray structure. He points out that these regions are about as dense as liquid benzene (0.88 g cm^{-3}). It is interesting to note, however, that the density of solid *n*-hexadecane is only 0.775 g cm^{-3} and the density of solid benzene is 1.01 g cm^{-3} at 0°, so that Kuntz' value can be regarded as quite a high one.

[7] The dependence of ΔV on pressure at constant temperature will be considered in more detail below.

there exists a potential contribution of about -7×35 ml, or about -245 ml/mol, to the volume change for denaturation due to the normalization of these basic groups. The corrected value of ΔV at 20° and near 1 atm given in Table II is approximately -100 ml/mol. If all of the charged basic side chains in metmyoglobin were normalized at acid pH, we would be left with an *expansion* of about 145 ml/mol ($= -100 + 245$) ascribable to the exposure of nonpolar groups to water. It is difficult to see how such a large volume change could arise from hydrophobic interactions alone even if the interior of the protein resembles a solid rather than a liquid.

On the other hand, at high pH we do not expect any contribution to the volume change for denaturation from the normalization of basic side chains, since these groups will not be charged at high pH. We have obtained an approximate value of -40 to -50 ml/mol for the volume change accompanying the unfolding of metmyoglobin at pH 10, 20°, 6000 kg/cm^2. Thus, after correction for the normalization of the basic side chains, the volume change accompanying the unfolding of metmyoglobin changes from $+145$ to -40 or -50 ml/mol as the pH is changed from 5 to 10. If this volume change is determined by the exposure of nonpolar groups to water, it is difficult to understand why it should depend so strongly on the pH.[8]

It is clear that the simple hydrophobic model does not adequately explain the behavior of proteins under pressure. Several reasons for this inadequacy are possible.

The estimates of the hydrophobic contribution to the volume change accompanying protein denaturation are based on the assumption that, upon denaturation, the exposed nonpolar groups are essentially at infinite dilution. Bøje and Hvidt (1971, 1972) have pointed out that the local concentration of nonpolar groups is quite high in a randomly coiled protein. They have investigated the effects of local concentration on the volume change expected from the rupture of hydrophobic bonds and show that because of this effect ΔV for this process could be positive in proteins. We do not have any knowledge of the pressure dependence of the concentration effects discussed by Bøje and Hvidt. It is therefore not possible to say whether or not these effects could account for all of the discrepancies between the volume changes observed for protein denaturation and those expected from model compound studies.[9]

Suzuki *et al.* (1970) have found from the effect of pressure on the water solubility of diketopiperazine, a model for the peptide bond, that the solubility passes through a minimum at 4500 kg/cm^2. These effects of pressure on the solubility are, however, much smaller than those found with nonpolar compounds in water because the volume changes are much smaller (at 1 atm, ΔV for the process diketopiperazine(s) → diketopiperazine (aqueous) is only $+5$ ml/mol, and ΔV goes to zero at 4500 kg/cm^2). If these volume changes are ascribed to the breaking of peptide–peptide hydrogen bonds then we can perhaps understand why the ΔV of unfolding remains negative at high pressures, and also why ΔV is not as large as anticipated at low pressures. For instance, if 10 nonpolar groups are exposed on denaturation (each contributing a volume change of -20 ml/mol at 1 atm and $+4$ ml/mol at 10,000 kg/cm^2) and if 24 peptide groups are exposed on denaturation (each contributing $+5$ ml/mol at 1 atm and -5 ml/mol at 10,000 kg/cm^2) then ΔV for unfolding would be -80 ml/mol at both 1 atm and 10,000 kg/cm^2. This would still not account for the failure to observe a rapid change in ΔV toward less negative values as the pressure is raised from 1 atm to 2000–3000 kg/cm^2, as expected from the hydrophobic model compound studies.

Dependence of Volume Changes on Pressure, Temperature, and pH. Despite the variation in v with pH and pressure, the corrected volume changes at 20° given in column 6 of Table II are all near -100 ml/mol. Nevertheless, small changes are seen when the pressure is varied from 600 to 6000 kg/cm^2 at pH 4–6. If these changes are real and are caused entirely by a dependence of ΔV on the pressure they would indicate a change in compressibility ($\Delta\kappa = (\partial V/\partial P)_T$) on denaturation of the order of -0.004 ml/(atm mol). Brandts *et al.* (1970) found $\Delta\kappa = +0.02$ for ribonuclease and Hawley (1971) found $\Delta\kappa = -0.03$ for chymotrypsinogen at constant pH. These values are opposite in sign and much larger than the value suggested here for metmyoglobin. It is, however, possible that the positive compressibility change observed by Brandts and Hawley is also present in metmyoglobin but is obscured by a pH dependence of ΔV not accounted for by the normal protonation of imidazole groups.

The results presented in the last seven rows of Table II (in which the temperature is varied) should yield information on the change in the coefficient of thermal expansion ($\Delta\alpha = (\partial\Delta V/\partial T)_P$) for the denaturation of metmyoglobin. At a pH near 5, the corrected volume changes given in Table II appear to be approximately constant over the temperature range 5–40°. The pressure is, however, not constant over this temperature range but varies by about 2000 kg/cm^2. Therefore, if we assume $\Delta\kappa = -0.004$ ml/(mol atm) (see above), then the volume change between 5 and 40° at pH near 5 should increase by about 8 ml/mol, which corresponds to $\Delta\alpha = +0.3$ ml/deg. Between 40 and 60° (pH ~5, pressure ~4100 kg/cm^2) ΔV changes markedly, becoming much less negative at the higher temperature. This change in ΔV corresponds to $\Delta\alpha = +2.4$ ml/deg, suggesting that there may be a large effect of temperature on $\Delta\alpha$ at pH 5. On the other hand, it is possible that $\Delta\alpha$ is also large between 5 and 40° but that its effect on ΔV is obscured by a large positive $\Delta\kappa$.

Behavior similar to that described above at pH ~5 can also be seen in the last three rows of Table II, which give ΔV as a function of temperature and pressure at pH ~5.7. At this pH, however, the change in ΔV between 5 and 40° is somewhat larger than observed at pH ~5 for the same temperature interval. Upon correction for a possible $\Delta\kappa$ of -0.004 ml/(atm mol), the change in ΔV between 5 and 40°, pH ~5.7, corresponds to a $\Delta\alpha$ of $+1.8$ ml/deg. This value of $\Delta\alpha$ is significantly larger than was indicated at pH ~5 between 5 and 40° and suggests that $\Delta\alpha$ may be somewhat pH dependent. It cannot, however, be ruled out that this apparent difference in $\Delta\alpha$ between pH 5 and 5.7 arises from some effect of pH on $\Delta\kappa$.

Hawley (1971) derived $\Delta\alpha$ values of $+1.32$ ml/deg from his data on chymotrypsinogen and $+0.252$ ml/deg from the data of Brandts *et al.* (1970) on ribonuclease. These values

[8] It is possible, of course, that the neutral form of the basic side chain is normalized on denaturation, rather than the charged form, as assumed above. If this were the case, then ΔV on unfolding at low pH would have the observed value of -100 ml/mol. The estimated value of -40 to -50 ml/mol at pH 10 would be corrected to -290 ml/mol ($= -45 - 245$) when normalization is taken into account. Again there is a large pH dependence of ΔV to be ascribed to the exposure of nonpolar groups to water.

[9] It should be pointed out that if there is indeed a significant effect of the local concentration of nonpolar groups on ΔV, then one might expect a significant effect on ΔC_p as well. If Bøje and Hvidt are correct, then the interpretation of the large value of ΔC_p that is often observed when proteins unfold may also have to be modified.

are of the same sign and order of magnitude as those suggested above for metmyoglobin.

Appendix

Estimation of the Temperature of Maximum Stability. Suppose that

$$\Delta H = \Delta H_0 + \Delta C_p(T - T_0)$$

$$\Delta S = \Delta S_0 + \Delta C_p \ln (T/T_0)$$

where ΔS_0, ΔH_0, and ΔC_p are independent of the temperature. Then

$$\Delta G = \Delta H_0 + \Delta C_p(T - T_0) - T\Delta S_0 - \Delta C_p T \ln (T/T_0)$$

The temperature of maximum stability is found by setting $d(\Delta G/T)/dT = 0$ which gives

$$T_{max} = T_0 - (\Delta H_0/\Delta C_p) \qquad (1')$$

Thus if ΔH_0 and ΔC_p are known, T_{max} can be estimated. The 1-atm results of Acampora and Hermans (1967) on metmyoglobin at pH 5 give $\Delta C_p \cong 2000$ cal/(deg/mol) and $\Delta H \cong 0$ at 25°, so that $\Delta H_0 = 0$ when $T_0 = 25°$ and $T_{max} = 25°$ at 1 atm.

Kliman's studies on a simple hydrophobic interaction model lead us to expect that ΔH_0 for proteins should become more positive by about 1500 cal per nonpolar group exposed when the pressure is raised by 2500 kg/cm². Furthermore, ΔC_p should decrease linearly with increasing pressure, becoming zero at about 5000 kg/cm², so that at 2500 kg/cm² ΔC_p might be expected to have half of its value at 1 atm, or about 1000 cal/(deg mol). If n nonpolar side chains are exposed to the solvent on denaturation then at 2500 kg/cm² we should have

$$T_{max} = 25° - (1500n/1000) = (25 - 1.5n)°C$$

Thus, according to eq 1′, the temperature of maximum stability of metmyoglobin at pH 5 should decrease with increasing pressure if the thermodynamic parameters ΔH_0 and ΔC_p behaved in the manner expected from the simple hydrophobic interaction model.

Supplementary Material Available

Supplementary material giving the data used to obtain the plots to which footnote 4 refers will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 20× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number BIO-73-4217.

Added in Proof

Katz *et al.* (1973) have used dilatometers to measure the volume changes accompanying the transition undergone by metmyoglobin at 30° when the pH is reduced from 4.3 to 4.0 by adding HCl. They obtain a value of −550 ml/10⁵ g of protein for the ΔV of this transition (after including the contribution due to the uptake of protons by carboxyl and imidazole groups in the transition). Since metmyoglobin has a molecular weight of 17,800, this corresponds to a ΔV of −98 ml/mol of protein, which is close to the values given in column 6 of Table II at pH 4–5, 20–40°. This agreement between the dilatometric ΔV and the value of ΔV obtained from the effect of pressure on K_{eq} supports the validity of the use of the two-state model in the interpretation of our results.

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Physicochemical and Biological Studies on the Metal-Induced Conformational Change in Troponin A. Implication of Carboxyl Groups in the Binding of Calcium Ion†

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ABSTRACT: The Ca^{2+}-induced conformational change in which troponin A assumes a more helical conformation has been explored in greater detail, using circular dichroism, molecular weight, fluorescence, and biological techniques. It has been demonstrated that other bivalent ions, such as Mg^{2+}, Ba^{2+}, and Cd^{2+}, are capable of inducing a similar type of change in the protein molecule, the effect, however, being only about 50–70% the magnitude of that produced by Ca^{2+}. A possible explanation for this finding is the much lower (~1/500) binding constants of these metals for troponin A, compared with Ca^{2+}. Circular dichroism studies extended to higher total ionic strength than before demonstrated that the Ca^{2+}-induced change was not an artifact of the initial relatively low ionic strength buffer system employed. Several derivatives of troponin A have been prepared in which a varying number of carboxyl groups have been replaced by uncharged glycinate residues. Circular dichroism and fluorescence studies indicated that these modified samples were affected only slightly by Ca^{2+} ions. Biological studies on these derivatives employing desensitized actomyosin, the Mg^{2+}-activated ATPase of which was partially inactivated by "inhibitory protein," indicated a great loss in their ability to overcome or neutralize the effect of inhibitory protein. At the same time, sedimentation equilibrium studies revealed no significant alteration in molecular weight or molecular weight behavior (*e.g.*, association–dissociation phenomena), compared to native troponin A. It is concluded that the main sites on the troponin A molecule available for interaction with Ca^{2+} are certain carboxyl groups of aspartic and glutamic acid residues. Removal of these regions of negativity allows the molecule to adopt a slightly different conformation, comparable to that assumed by the molecule when Ca^{2+} is added, and to become essentially insensitive to the presence or absence of Ca^{2+} ions.

The troponin complex of skeletal muscle is composed of three distinct proteins (Drabikowski *et al.*, 1971; Ebashi *et al.*, 1971; Greaser and Gergely, 1971; Wilkinson *et al.*, 1971; Murray and Kay, 1971). These are the 37,000-dalton component or TN-T,[1] the function of which is not understood, but which is believed to interact with tropomyosin; the inhibitor protein, TN-I, which inhibits the Mg^{2+}-activated ATPase activity of desensitized actomyosin; and finally, the calcium binding protein, TN-C or troponin A, which in the presence of Ca^{2+} ions neutralizes the effect of inhibitory protein.

It has been demonstrated that troponin A binds Ca^{2+} very strongly (Fuchs, 1971), with a binding constant of about 10^6 M^{-1} (Ebashi *et al.*, 1968; Hartshorne and Pyun, 1971). Upon binding Ca^{2+}, troponin A undergoes a remarkable conformational change, with no alteration in molecular weight (Murray and Kay, 1972; Van Eerd and Kawasaki, 1972). As this conformational change occurs over a physiological range of concentration of Ca^{2+} ions, it may well be involved in the ability of troponin A to neutralize the effects of inhibitory protein.

This study was initiated with a view to defining more precisely the molecular basis of the Ca^{2+}-induced conformational change in troponin A. The conformational studies cited above involved working at rather low ionic strength (~50 mM) and the possibility could not be excluded that the conformational change noted may be a partial artifact as a result of the Donnan effect. To eliminate this possibility, in the present study 0.15 M KCl was included in the solvent system, and circular dichroism and molecular weight measurements were employed to monitor any changes noted in troponin A upon

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[1] Abbreviations used are: TN-T, the 37,000-dalton component of the troponin complex, or tropomyosin binding factor; TN-I, inhibitory protein; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid; CD, circular dichroism.