HEME PROTEIN FLUORESCENCE VERSUS PRESSURE

M. C. MARDEN, G. HUI BON HOA, AND F. STETZKOWSKI-MARDEN
Institut de Biologie Physico-Chimique, 13, rue P et M Curie, 75005 Paris

ABSTRACT Fluorescence spectra of several ferric heme proteins have been measured vs. pressure to 6,000 bars. Sperm whale myoglobin (SW Mb), *Aplysia* myoglobin, leghemoglobin (Lb), and cytochrome P450 all show excitation and emission spectra characteristic of tryptophan in proteins with peak emission at 330–340 nm. At one bar, the fluorescence is weak due to energy transfer to the heme group, which makes the yield a sensitive probe of protein unfolding at high pressure. After an initial decrease of a few percent per kbar, the protein shows a large increase in fluorescence at high pressure. The increase is pH dependent and the results indicate that several high pressure states occur. For SW Mb at 15°C an increase of a factor of 20 occurs with midpoint at 2,000 bars at pH 5 and is only partially reversible, while the increase at pH 7 occurs at 4,000 bars and is only half as large and is completely reversible. *Aplysia* Mb and Lb show a similar effect, but unfold at a higher pressure than SW Mb. P450 also shows a transition to a state of higher fluorescence, but the transition in this case is irreversible as a stable form, P420, is formed. The fluorescence intensity measurements permit an estimation of the increase in the TRY-heme distance in the high pressure state.

The dominant fluorophore in proteins is the tryptophan residue (TRY), which on the average emits one photon for every five absorbed. The fluorescence yield depends on temperature, pressure, and the local environment which makes the fluorescence properties an excellent probe of the protein interior (Teale and Weber, 1957; Teale, 1960). In heme proteins, in addition to fluorescence and nonradiative pathways, there is energy transfer to the heme group which can greatly reduce the observed fluorescence yield and lifetime (Weber and Teale, 1959; Alpert et al., 1980; Hochstrasser and Negus, 1984). The energy transfer rate is proportional to the inverse sixth power of the distance between the donor and acceptor making the fluorescence yield highly sensitive to conformational changes. Heme proteins provide a natural donor-acceptor system, since the absorption spectrum of the heme group overlaps with the tryptophan emission in the 300–420 nm region.

The low fluorescence yield is sensitive to small changes in structure as seen in denaturation studies of *Aplysia* Mb[1] (Brunori et al., 1972) and cytochrome-*c* (Tsong, 1974). Removal of the heme group results in a yield for the APO-protein of approximately half that of free TRY (Lindqvist et al., 1978; Anderson et al., 1970). If only the iron atom is removed, the TRY fluorescence is still quenched, but the porphyrin emission can then be observed (Sebban et al., 1980).

Systems with energy transfer can provide information on small changes in the static and dynamic properties of the fluorescent probe (Stryer, 1978; Badley and Teale, 1969). As a static probe, the fluorescence intensity can be used to determine distance as in the mapping of components of cytochrome-*c* oxidase (Dockter et al., 1978). Changes in yield vs. temperature have been used to estimate the dynamics of labeled ribonuclease T_1 (Somogyi et al., 1984).

In this study, high pressure is used to induce conformational changes in several heme proteins. The initial effect in every case is a reduction of the yield by several percent. At higher pressures the protein will partially or completely unfold resulting in a large increase in yield. Since the energy transfer rate depends on many parameters, we first review the relevant Förster theory (1959) and then describe the heme proteins studied here.

The energy transfer rate is proportional to the inverse sixth power of the donor-acceptor distance resulting in an observed yield (Q) which is reduced by a factor

$$Q_a/Q = 1 + (R_o/R)^6. \quad (1)$$

Q_a is the yield in the absence of energy transfer and R is the distance between the donor and acceptor. The critical distance for 50% quenching R_o (in Å) is calculated as

$$R_o = 9{,}790(Q_a K^2 J/n^4)^{1/6}. \quad (2)$$

n is the index of refraction and J an overlap integral of the donor emission and acceptor absorbance (ϵ in M^{-1} cm^{-1}, λ in cm)

$$J = \int F(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda \Big/ \int F(\lambda) \, d\lambda. \quad (3)$$

K^2 is an orientation factor which can vary between 0 and 4 for two static vector dipoles, with an average value of 2/3 when the transition dipoles rotate on a time scale shorter than the fluorescence lifetime. Both the static and dynamic

M. C. Marden's present address is Cornell University, Biochemistry Department, Ithaca, NY 14853

[1] *Abbreviations in this paper*: Mb, myoglobin; SW Mb, sperm whale myoglobin; Lb, leghemoglobin; P450, cytochrome P450; APO-, protein after removal of heme; Å, angstrom = 0.1 nm; bar, 10^5 kg/ms^2; SDS, sodium lauryl sulfate; TRY, tryptophan.

properties are required to calculate K^2 (Dale et al., 1979)

$$K^2 = (\cos\theta_{ad} - 3\cos\theta_{dr}\cos\theta_{ar})^2, \quad (4)$$

with θ the angle between the vectors indicated by the subscripts d = donor, a = acceptor, and r = center to center.

The fluorescence intensity is sensitive to small changes in R or K^2. Consider for example a system with R_0 = 20 Å and R = 15 Å so the yield is reduced by a factor of 6.6. Then a perturbation of R of 0.1 Å causes a 3% change in observed yield. Such effects are easily measured; however, the interpretation requires consideration of several parameters. For a highly quenched system, $(R_0/R)^6 > 10$, Eqs. 1 and 2 show that the fluorescence intensity can be written as

$$F \propto R^6 n^4/(K^2 J) \quad (5)$$

The following effects are important for measurements vs. pressure.

(*a*) The compression of the solvent causes an increase in protein concentration. For water at 15°C the increase is 4% at 1kbar and 10% at 3 kbars. Self absorption (inner filter) effects will also increase.

(*b*) The yield in the absence of energy transfer may be pressure dependent. Free DL-TRY yield increases initially as 10% per kbar (before correction for solvent compression).

(*c*) Protein compression implies a change in R. If the compressibility of the protein is as large as that of the solvent, then the change in linear dimensions will be ~1% per kbar. This means a decrease of fluorescence of 5–6% per kbar for a highly quenched system. Changes in the index of refraction also need to be considered because n will change with the density.

(*d*) Changes in the acceptor absorption spectrum will effect the fluorescence; this effect may be large upon denaturation.

(*e*) Changes in either the static or dynamic properties of the donor or acceptor will effect the fluorescence yield. Upon complete unfolding, the TRY should rotate freely and K^2 will approach 2/3.

(*f*) The largest effect on the yield is the unfolding which causes a large change in R.

Ferric Heme Proteins

SW Mb was chosen due to the great amount of information available on the structure and stability. The first protein structure to be determined by x-ray crystallography, SW Mb has also been well studied for its ligand binding properties (Antonini and Brunori, 1971) and its stability vs. temperature, pressure, and pH (Zipp and Kauzmann, 1973). SW Mb has 153 residues with TRY at positions 7 and 14. Polarization studies of TRY fluorescence in APO-SW Mb indicate that the TRY residues are rigidly held (Anderson et al., 1970). Recently, time resolved fluorescence decays of SW Mb have been measured (Hochstrasser and Negus, 1984). Components were observed with decay times 170 and 20 times faster than the APO-SW Mb decay of 2.8 ns.

For comparison, two other species of Mb were studied; one is extracted from marine molluscs, the other from plants. *Aplysia* Mb lacks the distal histidine and has an oxygen affinity five times less than SW Mb. In the ferric acidic form, the iron is probably penta-coordinated; *Aplysia* -Mb may lack the water molecule seen in x-ray data of ferric, acidic SW Mb. The Soret band of *Aplysia* Mb is smaller and blue shifted relative to SW Mb, being intermediate to free heme and SW Mb. *Aplysia* and SW Mb show a red shift in Soret band with pK = 7.5 and 9, respectively.

Leghemoglobin, a monomeric hemoglobin found in the root nodules of leguminous plants, has an oxygen affinity 10 times higher than SW Mb. Its function could be to maintain a low oxygen level in the bacteroids where the nitrogenases operate. Although Lb is a functional and structural homologue of mammalian Mb, the two differ in over 85% of the amino acid residues and x-ray and ligand kinetics studies indicate that Lb has a larger heme pocket. Soybean Lb has 143 residues with TRY at 126 and 134.

Also studied here is the mono-oxygenase, cytochrome P450. Unlike Mb and Lb, P450's natural function is in both the ferric and ferrous states as it participates in oxygen binding, substrate binding, and a redox reaction (Sato, 1978). This protein of MW 45,000 is nearly three times as large as Mb or Lb, but shares many ligand binding properties. P450 appears to be more fragile than other heme proteins and loses its unique property of absorbance at 450 nm (CO bound) at high temperature or pressure. This transition and the spin change in the ferric form have previously been measured vs. pressure (Hui Bon Hoa and Marden, 1982; Marden and Hui Bon Hoa, 1982). The species with camphor as substrate has 412 residues with TRY at positions 42, 61, 372, and 404 (Hanui et al., 1982). The species with linalool as substrate has 6 tryptophans whose positions are unknown at this time.

The goal of the present work is to provide additional information on the high pressure states of these proteins. Absorption measurements have shown several types of states to exist for SW Mb (Zipp and Kauzmann, 1973). There may be large changes in absorbance, as in ligand binding, with only small changes in fluorescence. This means that the local electronic state of the heme may change without a large conformational change in the protein. Conversely, the partial unfolding of the protein may cause a several fold increase in the TRY fluorescence, while the Soret band is not significantly modified. The fluorescence work provides information on the TRY environment and the TRY-heme geometry and reflects a more global probe of the protein.

MATERIALS AND METHODS

Fluorescence measurements were made on an SLM-4800 spectrofluorometer with automatic wavelength control and correction for changes in lamp intensity. The machine was interfaced to a microcomputer (Sirius) for data accumulation and integration of spectra vs. inverse wavelength to determine relative yields. The phototube was an EMI 9813 with peak response near 380 nm; sensitivity was >90% of the peak between 320–440 nm. The monochromator was a holographic grating, SMC 230 (SLM). No additional corrections were made for the wavelength response of the detection system.

Yields were determined relative to free DL-tryptophan in a 0.4 × 1.0 cm quartz cuvette for samples of equal absorption at 280 nm of 0.1 OD/cm using exciting light at 280 nm along the 1 cm axis. The spectrum of the solvent was subtracted before comparison of the integrated intensities.

Excitation at 295 nm is often used to avoid absorption by the tyrosine residues, but this wavelength is on the steep slope of the TRY absorption curve. Slight shifts in the absorption spectrum would therefore cause changes in the fluorescence yield. The yields of the proteins relative to TRY were larger at higher wavelengths due to a 0–2 nm shift in the absorption spectrum. Variation of the excitation wavelength from 280–295 nm had little effect on the results vs. pressure for SW Mb; 286 nm was used for most experiments. 280 nm light caused some denaturation of P450, even at 1 bar, so 290 nm was used. The bandpass for both excitation and emission monochromators was 4 nm.

Yields were also measured after addition of sodium lauryl sulfate (SDS). 0.1% SDS, enough for protein denaturation, caused a 20% increase in yield in APO-SW Mb and a 10% decrease in yield of free TRY.

High Pressure Apparatus

The pressure system, capable of 6,200 bars, has been described previously (Hui Bon Hoa et al., 1982). The bomb is cubical, 11 cm on a side, with a copper jacket for temperature control. The bomb plus jacket fits snugly in the SLM sample compartment, thus minimizing any movement relative to the light paths. The high pressure windows are sapphire discs polished optically flat. Pressure is given in units of bar = 0.987 atm = 10^5 Pascal = 10^5 kg/ms^2 and corresponds to the hybrid unit of 1.02 kg/cm^2. The high pressure cuvette was a 0.5 ml quartz cylinder, 2 cm long, 0.5 cm inner diameter, with flattened sides. Fluorescence was detected at 90° to the exciting beam which entered the bottom of the cylinder. A teflon membrane separated the sample from the pressurizing fluid, pentan or heptan (Merck).

From the known compressibility of water at 15°C, the calculated sample concentrations relative to 1 bar are 1.044, 1.078, 1.106, 1.131, and 1.152 at 1, 2, 3, 4, and 5 kbars. Samples showed the expected increase in absorption (CARY-219) vs. pressure as determined at isosbestic points. The fluorescence yield of free tryptophan increased by 10%/kbar between 1 and 3 kbars, in agreement with published values (Li et al., 1976*a*). Proteins without energy transfer generally show an increase in yield vs. pressure (Li et al., 1976*a*, *b*).

Much information on TRY fluorescence has been accumulated. The quantum yield of free TRY is temperature dependent, rising from 0.14 at 27°C to nearly 0.6 at −80°C (Eisinger and Navon, 1969; Galley and Edelman, 1962). The yield in proteins may be higher or lower, but is typically within a factor of two of free TRY (Konev, 1967; Steiner and Weinryb, 1971). The yield of the APO-Mb of both sperm whale and *Aplysia* is 60% that of free TRY (Anderson et al., 1970), while the native proteins are greatly quenched (Brunori et al., 1968, 1970). From polarization studies, it was concluded that TRY may be rigidly held or may rotate rapidly within certain angular limits, typically 15° (Lakowicz et al., 1983).

The inner filter effects of SW Mb were determined by measuring the emission spetra vs. concentration. With 280 nm excitation, the peak emission vs. concentration was linear below 0.05 OD (at 280 nm), but deviated by 30% from the linear extrapolation at 0.2 OD. The maximum signal occurred at 0.3 OD. The deviations occur at lower absorption levels if detection is made in the Soret band. For a typical SW Mb sample of 0.1 OD at the excitation wavelength, the inner filter effects are estimated to cause a 10% increase in concentration to appear as an 8–9% increase in fluorescence at the wavelength of peak emission. The peak emission was used rather than integrated intensities for the low pressure part of the data (before denaturation). This avoids the subtraction of background solvent which is appreciable at the raman line of water and is not accurate in the Soret band where reabsorption is strongest.

Proteins

All proteins were studied in the ferric (Fe^{+3}) form. SW Mb (Sigma, type II) was purified by exchange ion chromatography on a column of carboxy methyl cellulose with an ionic strength and pH gradient between 10 mM sodium phosphate buffer at pH 5.8 and 20 mM disodium phosphate. The major fraction, the third band, was kept. The purified SW Mb at pH 7 had a ratio of 5.4 for the absorbance of the Soret at 409 nm to the UV peak at 280 nm. APO-SW Mb was prepared from Mb using the procedure of Rossi-Fanelli et al. (1958) by extraction of heme with acid-acetone at −20°C. APO-SW Mb showed an UV peak at 281 nm with a residual peak at 409 nm, ratio 0.07, indicating that at most 1.5% holoprotein remained.

Aplysia Mb was prepared from the buccal and stomach muscles according to Rossi-Fanelli and Antonini (1957) with the modifications of Wittenberg et al. (1965). The protein was purified by filtration on a column of Sephadex G75 with 50 mM potassium phosphate and 150 mM NaCl at pH 7.8. The variety studied here is *Aplysia punctata*, a mollusc found off the North Atlantic coast of France. Other varieties known are *Aplysia limacina*, a Mediteranean mollusc, which has 145 residues with TRY at positions 14 and 29 (Tentori et al., 1973) and *Aplysia kurodai* from the Japanese coast which has 144 residues with TRY at positions 14 and 128 (Shikama et al., 1982).

Leghemoglobin (Lb) was isolated from the root nodules of soybean (Glycine max, Altona variety) infected with Rhizobium Japonicum. The fraction Lb-a was used and was prepared according to the method of Appleby et al. (1975).

All the purified proteins were stored in liquid nitrogen after dialysis against 10 mM potassium phosphate at pH 7 and concentration by filtration on UM10 diaflo membranes.

Cytochrome P450 samples for two species, with camphor or linalool as substrate, were provided by the laboratory of Professor I. C. Gunsalus. Stock solutions were 1 mM protein with substrate attached and were stored at −80°C.

Tris buffer was used for pH > 7, potassium phosphate in the range pH 6.5–7, and sodium acetate for pH < 6. The pH values reported are those measured at 1 bar. The pH of acetate buffer decreases by 0.22 units per kbar; phosphate decreases by 0.33 units per kbar, while Tris increases by 0.02 units per kbar (Neuman et al., 1973).

RESULTS

Emission spectra of SW Mb at 15°C, pH 7.2-Tris buffer at the indicated pressures are shown in Fig. 1 for excitation at 286 nm. After a decrease of 2%/kbar (before correction for the solvent compression), there is a large increase in fluorescence intensity at pressures above 2,000 bars. At pH 7.2 the transition is completely reversible and 6,000 bars was not sufficient for a complete unfolding.

The ratio of fluorescence yields of free TRY to SW Mb was 120 at pH 7, while that of free TRY relative to APO-SW Mb was 2. Since the two tryptophan residues in SW Mb account for approximately one-third of the protein absorption at 280 nm, the effective TRY yield in SW Mb relative to APO-SW Mb is ~1/20. Thus an increase of a factor of 20 is expected for a complete unfolding and this

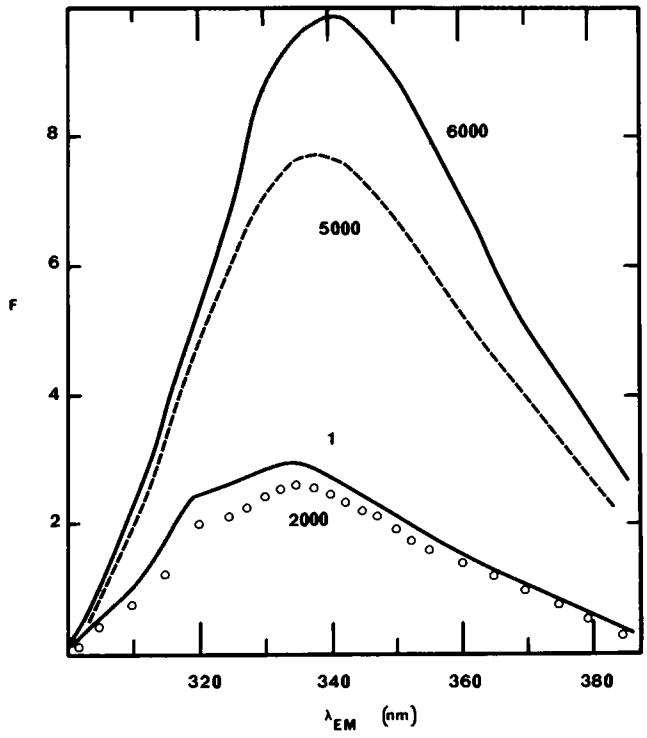

FIGURE 1 Fluorescence emission spectra of ferric SW Mb for 286 nm excitation at 15°C, pH 7.2 Tris buffer at the pressures indicated in bars.

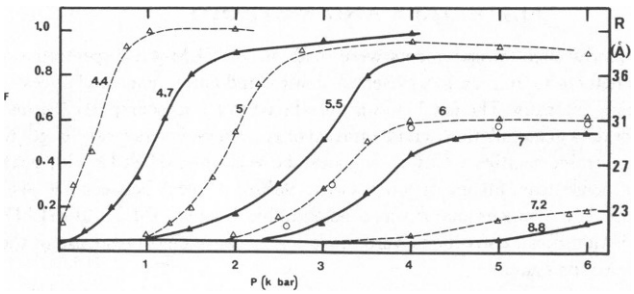

FIGURE 2 Relative fluorescence intensity at peak emission vs. pressure for SW Mb at 15°C at the pH values shown. Curves are normalized to the maximum observed yield (left hand scale). The scale on the right side gives values for the TRY-heme distance (R) using R_0 = 29 Å and K^2 = 2/3.

increase is observed after addition of SDS (factor of 21) or at high pressure on samples at low pH. The yields of proteins relative to free TRY were larger at 295 nm excitation than at 280 nm. This is due to the shift in the TRY absorption spectrum in proteins.

The fluorescence intensity at peak emission vs. pressure at several pH for SW Mb are shown in Fig. 2. The large increases occur at the same pressures that induced absorption changes in the Soret (Zipp and Kauzmann, 1973). The data are normalized to the maximum observed intensity, which occurs at 4,000 bars, pH 4.7 (or for SDS denatured protein). At low pH a transition of nearly a factor of 20 was observed; the intensity after the pressure cycle was typically twice the original intensity at 1 bar. The increase at higher pH is smaller and several pressure cycles at pH 7 showed no sign of denaturation.

Since the structure of SW Mb is known, the theoretical yield can be calculated. The overlap integral J was calculated to be 5.2×10^{-14} cm^3 M^{-1}, using the APO-SW Mb fluorescence spectrum and the absorption of SW Mb normalized at 409 nm to 157 mM^{-1} cm^{-1}. The overlap integral for heme proteins has a large contribution at both the peak of the fluorescence and at the Soret peak resulting in a wide overlap between 340 and 410 nm.

Since absorption occurs along both axes within the heme plane (Eaton and Hochstrasser, 1968), calculation of K^2 must consider transfer from the TRY transition dipole (Yamamoto and Tanaka, 1972) to both heme transitions. Using a coordinate system with the heme group in the x–y plane and the pyrrole nitrogens along the axes, the TRY-7 transition dipole is then in the x–z plane at an angle 40° from the z axis. The static K^2 for TRY 7 is ~1.3.

With n = 1.5, Q_a = 0.1, and the values for K^2 and J mentioned above, R_0 was calculated to be 32.5 Å (29 Å if K^2 = 2/3). This implies that the fluorescence is reduced by a factor of 13 for TRY-7 (R = 21.4 Å, center to center) and over 100 for TRY-14 (R = 15 Å), which is consistent with the observed yields and the lifetime measurements of Hochstrasser and Negus (1984). A change in fluorescence of 10% is expected for a change in R of 0.3 Å or a change of 3.5° in the angle between the TRY transition dipole and the z-axis (perpendicular to the heme).

At low pH the fluorescence at high pressure approaches the value in the absence of energy transfer. The left hand vertical axis of Fig. 2 is normalized to the maximum observed intensity. At pH > 6, the fluorescence is still reduced by at least a factor of two after the pressure transition. The vertical axis on the right hand side of Fig. 2 gives the value of R required for the yield. R was calculated with K^2 = 2/3, since the TRY is probably free to rotate after partial unfolding. This is an oversimplification, but is only intended to estimate the changes in R required to fit the changes in yield.

The data in Fig. 2 show that acetate pH 6 and phosphate pH 6.5 have nearly the same results (curve labeled 6). This is due to the pressure dependence of the pH of these buffers. Since pH decrease with pressure for phosphate and acetate buffers, the curves for pH < 7 are sharpened. The transition pressures and effective pH are summarized in Table I.

A comparison of SW Mb, *Aplysia* Mb, and Lb at pH 4.7 is shown in Fig. 3. The data show that both *Aplysia* Mb and Lb are more stable than SW Mb with respect to pressure denaturation in this region. The data are normalized to the maximum observed yield for each protein as in Fig. 2.

Fig. 4 shows that addition of KCN to SW Mb at pH 7, forming the Fe^{+3}-CN complex, results in a more stable system. However, removal of the heme group destabilizes the globin. These effects have been seen in the case of

TABLE I
HIGH PRESSURE FLUORESCENCE OF FERRIC HEME PROTEINS

molecule	pH-buffer	P_d (kbar)	pH at P_d	$\frac{Q}{Q\text{-TRY}}$	$\frac{Q\ (6\ \text{kbar})}{Q\ (1\ \text{bar})}$
TRY	7.2-Tris	>6	7.2	1	1.5
SW Mb	4.4-Acet	0.4	4.3	0.012	15
	4.7-Acet	1.1	4.5		20
	5.0-Acet	2	4.6	0.01	20
	5.5-Acet	3	4.8		18
	6.0-Acet	3.5	5.2		12
	6.5-Phos	3.5	5.3		12
	7.0-Phos	4.2	5.6	0.0083	11
	7.2-Tris	>6	7.3		5
	8.8-Tris	>6	8.9		2
APO-Mb	7.0-Phos	1.5	6.5	0.5	1.6
Mb-CN	7.0-Phos	5	5.4	0.01	10
Aplysia Mb	4.7-Acet	4	3.8		2.2
	6.0-Acet	5	4.9		2
	7.2-Tris	6	7.3	0.1	1.3
	8.8-Tris	>6	8.9		1.3
Mb-CN	7.2-Tris	>6	7.3		1.2
Leghemoglobin	4.7-Acet	4.5	3.7		5.5
	5.5-Acet	5.5	4.3		2.2
	7.2-Tris	>6	7.3	0.02	1.0
	8.8-Tris	3.5	8.8		1.3
P450-camphor	7.2-Tris	1.2	7.2	0.04	1.6
P450-linalool	7.2-Tris	3.5	7.3	0.07	1.6

The yield at 1 bar relative to free TRY (Q/Q-TRY) is for equal absorbance at 280 nm with excitation at 280 nm. P_d is the pressure of the midpoint of the transition.

fluoride or cyanide binding in absorption studies (Ogunmola et al., 1977) and in temperature denaturation of APO-SW Mb (Antonini and Brunori, 1971). SW Mb showed an increased stability as the temperature was raised from 4 to 25°C, in agreement with absorption studies (Zipp and Kauzmann, 1973); however, APO-SW Mb was more stable at lower temperatures. APO-SW Mb showed a 60% increase in fluorescence, which means the yield at high pressure is 80% that of free TRY. Note that a

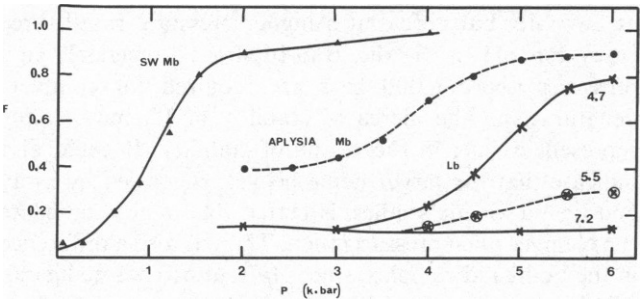

FIGURE 3 Relative fluorescence intensity vs. pressure at 15°C for Lb-a at the pH values shown and comparison to SW Mb and *Aplysia* Mb at pH 4.7. All proteins were in the ferric state.

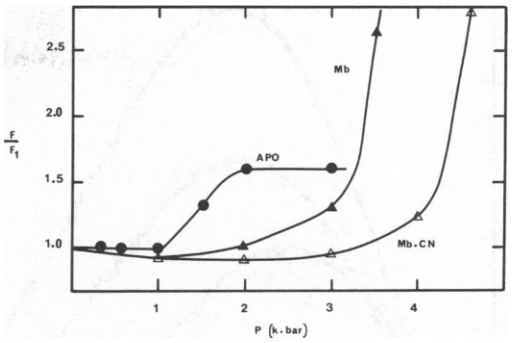

FIGURE 4 Fluorescence intensity relative to one bar (F_1) vs.pressure at 15°C, pH 7 for SW Mb, SW Mb-CN, and APO-SW Mb. Each curve was normalized to 1 at one bar.

different normalization is used in Fig. 4 to show SW Mb and APO-SW Mb on the same plot. The values are with respect to the yield at 1 bar (F_1) for each sample.

P450 is active only in the narrow pH region of 5.5–7.5 and easily decays to the inactive form P420. Emission spectra of P450-camphor at pH 7 are shown in Fig. 5. As the pressure is initially increased, there is a reversible decrease in yield of 5%/kbar (curve *A* to *B* in Fig. 6). As the pressure is further increased there is a 60% increase in fluorescence which requires nearly 1 h for equilibrium, (plateau *C*). The transition is nearly complete by 2,000 bars and still higher pressures cause a decrease in yield. Upon release of the pressure, the curve follows a new path (*D*). After the pressure cycle, the protein shows an absorption peak at 421 nm upon reduction with dithionite in the presence of CO. This form, P420, shows a large decrease in yield vs. pressure, but appears to be stable to at least 6,000 bars. The increase in yield after addition of SDS to P450-camphor is a factor of 4. The normalization in Fig. 6 is with respect to values at 1 bar.

Also shown in Fig. 6 are data for P450-linalool. The sequence of events is the same, but a higher pressure is required to make the transition to P420. This species also shows a weaker dependence of the spin equilibrium vs. pressure, which is now found to be correlated with the substrate binding. The form without substrate for both species denatures at a much lower pressure.

All the heme proteins studied had a red shift of 5 to 10 nm in the emission spectrum accompanying the large increase in yield at high pressure. This shift is in the direction of free TRY. There was no shift in the low pressure region (before denaturation), where the yield shows a small decrease.

Kinetics of the pressure denaturation were observed at the peak emission. Sample kinetics after a pressure jump are shown in Fig. 7 for SW Mb at pH 5. There was an initial decrease in rate vs. pressure. Beyond 50% denaturation, the rate then increases with pressure. A similar trend was observed for *Aplysia* Mb and Lb. The time constant had a maximum value near 400 s near the midpoint of the

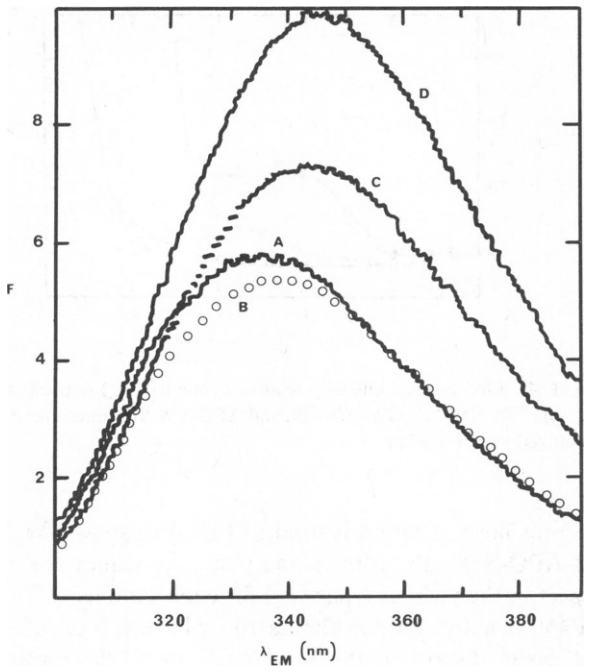

FIGURE 5 Fluorescence emission spectra of P450-camphor at 15°C, pH 7.2 with 286 nm excitation. Letters indicate the following states: A = 1 bar before pressurization, B = 900 bars during initial increase in pressure, C = 1,200 bars after 1 h, and D = 1,200 bars after a maximum pressure of 3,000 bars.

transition, independent of the protein (except P450) and pH. Time constants for the denaturation were in the range 100–400 s for the holo-proteins, while the APO-SW Mb denatured within seconds.

DISCUSSION

Fluorescence vs. pressure serves as a sensitive probe of heme protein conformational changes and denaturation. The results indicate that at least three types of denaturation must be considered. The first is the irreversible change of state observed in P450. This transition is chemically reversible (Sato, 1978) and therefore does not represent a

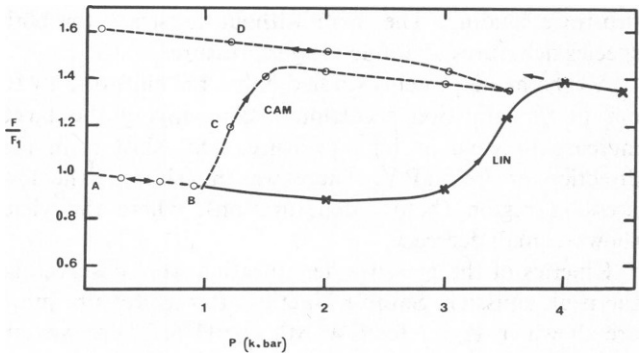

FIGURE 6 Fluorescence relative to that at one bar vs. pressure for P450-camphor (---) and P450-linalool (—) at 15°C, pH 7.2. Points labeled refer to spectra in Fig. 5. The large increase in intensity is not reversible.

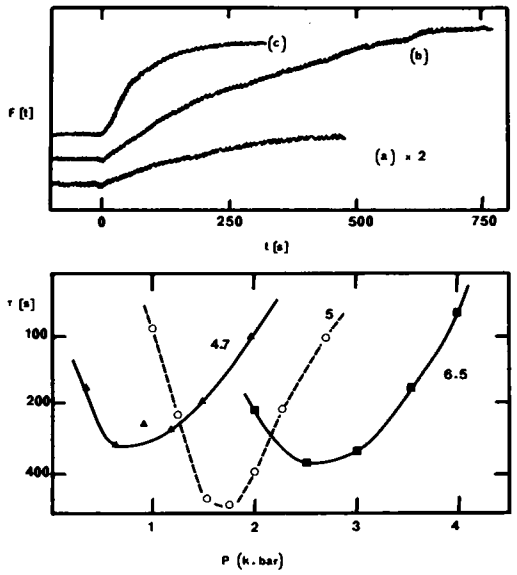

FIGURE 7 *Top*: Fluorescence intensity vs. time after pressure jump for SW Mb at 15°C, pH 6. Jumps are 1,700–2,000 (*a*), 2,000–3,000 (*b*), and 3,000–4,000 bars (*c*). *Bottom*: log of the time constant for unfolding vs. pressure.

loss of the protein. The increase in yield of 60% is small compared to that for unfolding with SDS (factor of 4), so the "denatured" form P420 is not completely unfolded.

A second form is that observed for Mb and Lb at pH > 6. This transition is completely reversible and incomplete relative to fluorescence levels obtained at low pH or with SDS indicating that the protein is partially unfolded. The results vs. pH (Fig. 2) show that several plateau values of the fluorescence may be obtained. The presence of several high pressure forms is consistent with the pH dependent Soret bands observed at high pressure (Zipp and Kauzmann, 1973).

A third form of denaturation observed is the partially reversible unfolding of Mb or Lb at low pH. The fluorescence in this case approaches that of SDS denatured protein or APO-protein. Since the fluorescence reaches the value expected in the absence of energy transfer, there is an increase in TRY-heme distance of at least 15 Å indicating a complete unfolding or loss of the heme group.

Aplysia Mb and Lb show the same trends and kinetics as SW Mb, but unfold at a higher pressure. In all three cases for pH > 6, the transition was reversible and pressures above 6,000 bars are required for complete denaturation. The increased stability of Lb may simply represent a shift in the region of stability. It could also indicate that the larger heme pocket, suggested by x-ray and ligand kinetic studies, is better able to accommodate the changes upon pressurization. There is also a difference in the Fe-ligand complex, since *Aplysia* Mb has no ligand, SW Mb has water, and Lb has acetate (in acetate buffer).

The species of P450 with linalool as substrate is more stable than the species with camphor. This is probably because camphor is the bulkier substrate and pressure

makes binding less favorable. Both species are less stable without the substrate.

It was also observed that glycerol prevents denaturation to higher pressure, as previously observed by absorption studies on P450. For both SW Mb and P450, glycerol favors the spectral form that occurs in water at high pH.

The kinetics of unfolding were similar for Mb and Lb. The rate was most strongly correlated with the state (fraction unfolded) of the protein, rather than the pH or pressure. The time constant was 100 s near 10% completion of unfolding, but increased to 400 s at the midpoint of the transition. This trend then reversed and a time constant of 100 s was observed near the state of 90% unfolded. This suggests a more universal rate for protein unfolding, rather than a protein specific rate. However, the irreversible transition of P450 (a larger protein) required 1 h and APO-SW Mb denatured within seconds.

There are in general two pressure regions to be considered: the high pressure unfolding and the compression prior to unfolding. The high pressure region will be discussed first.

The increase in yield vs. pressure for SW Mb is a factor of 20 at low pH, addition of SDS at pH 7 causes an increase of a factor of 21. The data can be fit by a simple two state model: folded with yield $Q_a/20$ and unfolded with the yield in the absence of energy transfer Q_a. The left hand axis in Fig. 2 can then be roughly read as the fraction unfolded for pH < 5.5.

At higher pH, a high pressure state is formed that still shows a reduced yield by at least a factor of 2. In this case values of the TRY-heme distance can be estimated for the partially folded state. R can be calculated given R_0 and the observed yield. This requires an assumption for the orientation factor K^2. For fixed K^2, a 50% increase in fluorescence would require an increase in R from 21.4 to 23 Å, while a twofold increase (to 0.1 in Fig. 2) corresponds to a 3.4 Å increase in R. The data near pH 6–7 that increase in yield of more than a factor of 10 therefore imply changes of nearly 8 Å in R. It is therefore probable that K^2 will also change; a value of 2/3 is expected if the TRY is free to rotate. The right hand vertical axis in Fig. 2 was calculated with $K^2 = 2/3$.

These large changes in R are still small compared to the separation based on a random polypeptide chain. From Tanford (1968), the average distance between residues separated by n amino acids is $(80n)^{1/2}$, which gives distances between histidine-64 (bound to the iron atom) and TRY-7 of 67.5 Å and TRY-14 of 63 Å.

Compressibilty

The low pressure region shows a decrease in fluorescence yield for all the heme proteins observed. This might represent an increase in energy transfer due to the protein compressibility (decrease in R). The same effect can be seen in the data of Li et al. (1976*a*) for the system with the energy acceptor, but not without the acceptor. Protein compressibilities have been calculated from sound velocity data giving values much smaller than for the solvents (Gekko and Noguchi, 1979). However, it was suggested that there is a negative contribution due to the hydrophobic interaction at the protein surface which is responsible for the net negative compressibilities of the free amino acids. The true protein compression, based on interatomic distances, may be much larger.

The decrease in yield before pressure denaturation is shown in Fig. 8. The decreases are on the order of 1–10%, which are easily measured, but requires consideration of several parameters in the energy transfer theory. Certain parameters may be correlated, such as R and n, since a smaller R due to a compressed protein implies a higher density and index of refraction. We first consider the combined effects of solvent and protein compressibilities. From Eq. 5, $F \propto R^6 n^4/V_s \propto V_p^2 n^4/V_s$ with V_p the protein volume and V_s the bulk solvent volume. The fractional change in fluorescence vs. pressure is then

$$\frac{1}{F} \cdot \frac{\partial F}{\partial P} = 2\beta_p - 4\gamma_n - \beta_s \qquad (6)$$

with $\beta = (-1/V)\,\partial V/\partial P$ the compressibility of the protein (p) or solvent (s) and γ_n the corresponding term for the index of refraction. Values of n and its pressure dependence in the protein are not known, but data for pure solvents indicate that $\gamma \approx \beta/4$ (Reisler and Eisenberg, 1965). Thus to a first approximation the pressure dependence of Eq. 6 reduces to $\beta_p - \beta_s$. This form provides a simple test of the relative compressibilities. For an incompressible protein, $\beta_p \ll \beta_s$ the data would follow the curve for solvent contraction (V_0/V) in Fig. 8), an increase in yield of 3%/kbar. For equal compressibilities, the effects should nearly cancel and there should be no observed change in the fluorescence. For $\beta_p > \beta_s$ the fluorescence intensity decreases vs. pressure. The data suggest that heme proteins are more compressible than water, but no

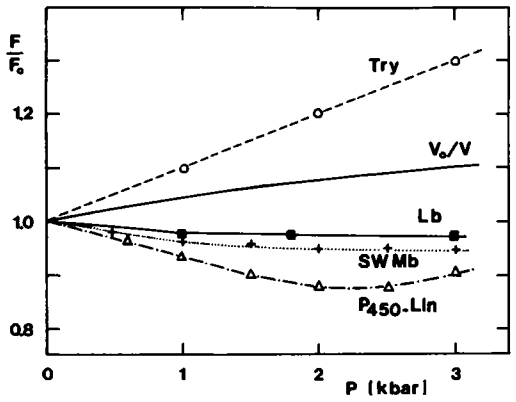

FIGURE 8 Fluorescence intensity vs. pressure for free DL-TRY, Lb-a, SW Mb, and P450-linalool at 15°C, pH 7 in the region before denaturation. Systems with no pressure dependence on their fluorescence properties would show only the increase due to solvent contraction (curve V_0/V).

precise calculations can be made due to the effects considered below.

Rotation of the TRY transition dipole towards the axis perpendicular to the heme plane would cause the yield to decrease by 3%/degree. Thus a rotation of a few degrees per kbar could also explain the data. Rotations toward the other axes would cause a much smaller change. Changes in the amplitude of dynamic motions about a fixed orientation cause a small change, since the changes in orientation relative to the heme perpendicular will nearly cancel. In general, changes in K^2 are expected to be both positive and negative, while the data show a decrease in fluorescence intensity for all the systems with energy transfer.

Another consideration is changes in the donor emission and acceptor absorbance spectra. These changes may be substantial for the denaturation, but are small prior to the transition.

A final consideration is the pressure dependence of the system in the absence of energy transfer. Free TRY shows a large increase in yield vs. pressure, which would seem to imply the compressibility effects are even larger. The yield in the absence of transfer is $Q_a = k_F/(k_F + k_R)$, where k_F is the intrinsic fluorescence rate and k_R the rate for radiationless decay. With an energy transfer rate k_T, the overall yield is $Q = k_F/(k_F + k_R + k_T)$, with k_T proportional to k_F. The stronger the fluorescence rate is, the larger the energy transfer rate in the presence of an acceptor. In the case of a system with a low yield due to energy transfer, changes in the total yield Q due to changes in Q_a will also be reduced.

Thus if free TRY were the correct reference state for SW Mb, the factor of 13 reduction in yield of TRY-7 would reduce the 4%/kbar change observed for Q_a of free TRY to <0.4%/kbar in Q. The effect of the other parameters can be considered without requiring a reference state such as free TRY or the system without energy transfer APO-protein.

The present estimates of protein compressibility are much larger than those reported using sound velocity techniques (Gekko and Noguchi, 1979; Gavish et al., 1983). Both techniques involve several contributions to the observed signal and require a separation of the relevant parameters. The sound velocity measurements need an estimate of the negative contribution of complicated protein solvent interface. Furthermore, the sound velocity technique measures an average protein value, whereas the fluorescence measurements refer to the TRY-heme distance.

Conclusions

Fluorescence of heme proteins can detect conformational changes as small as tenths of angstroms. The dominant signal vs. pressure is a large increase in yield upon unfolding with a time constant of 100–400 s for Mb and Lb. The initial decrease in yield suggests a protein compressibility comparable to that of the solvent.

Cytochrome P450 shows an irreversible transition to a state still reduced in yield by a factor of 2–3. The camphor system has the bulkier substrate and is more sensitive to pressure.

Aplysia Mb and Lb require a higher pressure for unfolding than for SW Mb. In all three systems, there are several unfolded states depending on the pH. The data show that above pH 6, high pressure states exist with the tryptophans displaced from their original positions by several angstroms.

We thank Professors R. Banerjee, P. Douzou, and I. C. Gunsalus for their support and useful discussions. We thank Dr. G. Wagner and Professor Gunsalus for supplying samples of both species of P450.

Received for publication 21 June 1985 and in final form 4 November 1985.

REFERENCES

Alpert, B., D. M. Jameson, and G. Weber. 1980. Tryptophan emission from human hemoglobin and its isolated subunits. *Photochem. Photobiol.* 31:1–4.

Anderson, S. R., M. Brunori, and G. Weber. 1970. Fluorescence studies of *Aplysia* and sperm whale apo-myoglobins. *Biochemistry.* 9:4723–4729.

Antonini, E., and M. Brunori. 1971. Hemoglobin and Myoglobin in Their Reactions with Ligands. North Holland Publishing Co., Amsterdam.

Appleby, C. A., N. A. Nicolas, J. C. R. Hurrel, and J. S. Leach. 1975. Characterization and improved separation of soybean leghemoglobins. *Biochemistry.* 14:4444–4450.

Badley, R. A., and F. W. J. Teal. 1969. Resonance energy transfer in pepsin conjugates. *J. Mol. Biol.* 44:71–88.

Brunori, M., G. M. Giacometti, E. Antonini, and J. Wyman. 1972. Denaturation of *Aplysia* myoglobin: equilibrium study. *J. Mol. Biol.* 63:139–152.

Brunori, M., E. Antonini, P. Fasella, J. Wyman, and A. Rossi-Fanelli. 1968. Reversible thermal denaturation of *Aplysia* myoglobin. *J. Mol. Biol.* 34:497–504.

Dale, R. E., J. Eisinger, and W. E. Blumberg. 1979. The orientational freedom of molecular probes: the orientation factor in intramolecular energy transfer. *Biophys. J.* 26:161–194.

Dockter, M. E., A. Steinemann, and G. Schatz. 1978. Mapping of yeast cytochrome-*c* oxidase by fluorescence resonance energy transfer. *J. Biol. Chem.* 253:311–317.

Eaton, W. A., and R. M. Hochstrasser. 1968. Single-crystal spectra of ferrimyoglobin complexes in polarized light. *J. Chem. Phys.* 49:985–995.

Eisinger, J., and G. Navon. 1969. Fluorescence quenching and isotope effect of tryptophan. *J. Chem. Phys.* 50:2069–2077.

Förster, Th. 1959. Transfer mechanism of electronic excitation. *Disc. Far. Soc.* 27:7–17.

Gally, J. A., and G. M. Edelman. 1962. The effect of temperature on the fluorescence of some aromatic amino acids and proteins. *BBA.* 60:499–509.

Gavish, B., E. Gratton, and C. J. Hardy. 1983. Adiabatic compressibility of globular proteins. *Proc. Natl. Acad. Sci. USA.* 80:750–754.

Gekko, K., and H. Noguchi. 1979. Compressibility of globular proteins in water at 25°C. *J. Phys. Chem.* 83:2706–2714.

Hanui, M., L. G. Armes, K. T. Yasunoba, B. A. Shastry, and I. C. Gunsalus. 1982. Amino acid sequence of the *Pseudomonas putida* cytochrome P450. *J. Biol. Chem.* 257:12657–12667.

Hochstrasser, R. M., and D. K. Negus. 1984. Picosecond fluorescence decay of tryptophans in myoglobin. *Proc. Natl. Acad. Sci. USA.* 81:4399–4403.

Hui Bon Hoa, G., P. Douzou, N. Dahan, and C. Balny. 1982. High pressure spectrometry at subzero temperatures. *Analyt. Biochem.* 120:125–135.

Hui Bon Hoa, G., and M. C. Marden. 1982. The pressure dependence of the spin equilibrium in camphor-bound ferric cytochrome P450. *Eur. J. Biochem.* 124:311–315.

Konev, S. V. 1967. Fluorescence and Phosphorescence of Proteins and Nucleic Acids. Plenum Publishing Corp., New York.

Lakowicz, J. R., B. P. Maliwal, H. Cherek, and A. Balter. 1983. Rotational freedom of tryptophan residues in proteins and peptides. *Biochemistry.* 22:1741–1752.

Li, T. M., J. W. Hook, H. G. Drickamer, and G. Weber. 1976*a*. Effects of pressure upon the fluorescence of the riboflavin binding protein and its flavin mononucleotide complex. *Biochemistry.* 15:3205–3211.

Li, T. M., J. W. Hook, H. G. Drickamer, and G. Weber. 1976*b*. Plurality of pressure denatured forms in chymotrypsinogen and lysozyme. *Biochemistry.* 15:5571–5580.

Lindqvist, L., A. Lopez-Campillo, and B. Alpert. 1978. Fluorescence properties of human adult apohemoglobin and its subunits. *Photochem. Photobiol.* 28:417–420.

Marden, M. C., and G. Hui Bon Hoa. 1982. Dynamics of the spin transition in camphor-bound ferric cytochrome P450 vs. temperature, pressure, and viscosity. *Eur. J. Biochem.* 129:111–117.

Neuman, R. C., W. Kauzmann, and A. Zipp. 1973. Pressure dependence of weak acid ionization in aqueous buffer. *J. Phys. Chem.* 77:2687–2691.

Ogunmalo, G. B., A. Zipp, F. Chen, and W. Kauzmann. 1977. Effects of pressure on visible spectra of complexes of myoglobin, hemoglobin, cytochrome *c*, and horseradish peroxidase. *Proc. Natl. Acad. Sci. USA.* 74:1–4.

Reisler, E., and H. Eisenberg. 1965. Refractive indices and piezo-optic coefficient of deuterium oxide, methanol, and other pure liquids. *J. Chem. Phys.* 43:3875–3880.

Rossi-Fanelli, A., and E. Antonini. 1957. A new type of myoglobin isolated and crystallized from the muscles of *Aplysiae. Biochemistry (Biokhimiya).* 22:312–321.

Rossi-Fanelli, A., E. Antonini, and A. Caputo. 1958. Studies on the structure of hemoglobin I. Physicochemical properties of human globin. *BBA.* 30:608–615.

Sato, R., and T. Omura, editors. 1978. Cytochrome P-450. Academic Press, Inc., New York.

Sebban, P., M. Coppey, B. Alpert, L. Lindqvist, and D. M. Jameson. 1980. Fluorescence properties of porphyrin-globin from human hemoglobin. *Photochem. Photobiol.* 32:727–731.

Shikama, K., T. Suzuki, Y. Sugawara, T. Katagiri, T. Takagi, and M. Hatano. 1982. *Aplysia* myoglobin with an unusual heme environment. *BBA.* 701:138–141.

Somogyi, B., J. Matko, S. Papp, J. Hevessy, G. R. Welch, and S. Damjanovich. 1984. Forster type energy transfer as a probe for changes in local fluctuations of the protein matrix. *Biochemistry.* 23:3403–3411.

Steiner, R. F., and J. Weinryb, editors. 1971. Excited states of proteins and nucleic acids. Plenum Publishing Corp., New York.

Stryer, L. 1978. Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* 47:819–846.

Tanford, C. 1968. Protein denaturation. *Adv. Prot. Chem.* 23:121–282.

Teale, F. W. J. 1960. The ultraviolet fluorescence of proteins in neutral solution. *Biochem. J.* 76:381–388.

Teale, F. W. J., and G. Weber. 1957. Ultraviolet fluorescence of the aromatic amino acids. *Biochem. J.* 65:476–482.

Tentori, L., G. Vivaldi, S. Carta, M. Marinucci, E. Antonini, and M. Brunori. 1973. The amino acid sequence of myoglobin from the mollusc *Aplysia limacina. Int. J. Peptide Protein Res.* 5:187–200.

Tsong, T. Y. 1974. The Trp-59 fluorescence of ferricytochrome *c* as a sensitive measure of the over-all protein conformation. *J. Biol. Chem.* 249:1988–1990.

Weber, G., and F. W. J. Teale. 1959. Electronic energy transfer in haem proteins. *Disc. Far. Soc.* 27:134–139.

Wittenberg, B. A., M. Brunori, E. Antonini, J. Wittenberg, and J. Wyman. 1965. Kinetics of the reactions of *Aplysia* myoglobin with oxygen and carbon monoxide. *Arch. Biochem. Biophys.* 111:576–582.

Yamamoto, Y., and J. Tanaka. 1972. Polarized absorption spectra of crystals of indole and its related compounds. *Bull. Chem. Soc. Japan.* 45:1362–1366.

Zipp, A., and W. Kauzmann. 1973. Pressure denaturation of metmyoglobin. *Biochemistry.* 12:4217–4228.