Thach, R. E. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y. Harper and Row, p 520.
Thach, R. E., and Doty, P. (1965), *Science 148*, No. 3670, 632.
Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 697.
Uhlenbeck, O. C., Baller, J., and Doty, P. (1970), *Nature (London) 225*, 508.
Uhlenbeck, O., Harrison, R., and Doty, P. (1968), *in* Molecular Associations in Biology, Pullman, B., Ed., New York, N. Y., Academic, p 107.
Warshaw, M. M., and Tinoco, I., Jr. (1965), *J. Mol. Biol. 13*, 54.
Wells, R. D., Jacob, T. M., Narang, S. A., and Khorana, H. G. (1967), *J. Mol. Biol. 27*, 237.
Yang, J. T., and Samejima, T. (1969), *Progr. Nucl. Acid Res. 9*, 224.

Fluorescence Studies of *Aplysia* and Sperm Whale Apomyoglobins*

Sonia R. Anderson,† Maurizio Brunori, and Gregorio Weber

ABSTRACT: We have investigated some of the molecular properties of the globins prepared from *Aplysia* myoglobin, sperm whale myoglobin, and human hemoglobin using fluorescence and fluorescence polarization methods. The study of the intrinsic fluorescence of the apomyoglobins shows definite differences between the two proteins. The fluorescence spectrum of *Aplysia* apomyoglobin clearly reveals two different tryptophan residues with emission maxima at *ca.* 330 and 355 nm. These wavelengths correspond, respectively, to the maxima of tryptophan in nonpolar and in aqueous solvents. The fluorescence polarization spectrum of *Aplysia* apomyoglobin demonstrates localized rotational freedom expected for a mobile tryptophan side chain in contact with the water. In contrast, the two tryptophan side chains of sperm whale apomyoglobin are rigidly bound in similar nonpolar regions within the protein matrix. The average lifetime of the excited state is in the range 2.8–2.9 nsec for both apomyoglobins. The fluorescence emission maximum of adsorbed 1-anilino-8-naphthalenesulfonate occurs at 478 nm in *Aplysia* apomyoglobin and at 455 nm in sperm whale apomyoglobin. This difference suggests that the specific dye binding site, believed to be the site normally occupied by the heme moiety of myoglobin, is more polar in *Aplysia* apomyoglobin. The rotational relaxation time obtained from measurements on conjugates with 1-dimethylaminonaphthalene-5-sulfonyl chloride is 30 nsecs at 15° for each of the apomyoglobins. This value indicates approximately spherical symmetry of the apomyoglobin molecule.

Sperm whale and *Aplysia* myoglobins have in common some of the most important structural and functional features such as molecular weight and ligand-binding properties (Rossi-Fanelli *et al.*, 1958b; Wittenberg *et al.*, 1965). On the other hand, they differ markedly in amino acid composition. Of particular interest in *Aplysia* myoglobin is the absence of tyrosine and the presence of only one histidine residue per molecule (Rossi-Fanelli *et al.*, 1958b; Tentori *et al.*, 1968). It is known that the amino acid sequence of the peptide containing the only histidine is considerably different from that of the peptides containing the proximal or distal histidine residues of sperm whale myoglobin (Tentori *et al.*, 1968). Finally, *Aplysia* myoglobin undergoes a reversible thermal denaturation involving the heme–protein interactions (Brunori *et al.*, 1968a).

The work reported here deals with some of the molecular properties of the apoproteins from *Aplysia* and sperm whale myoglobins as revealed by a study of the fluorescence and fluorescence polarization of the globins and of their conjugates with 1-dimethylaminonaphthalene-5-sulfonyl chloride and adsorbates with 1-anilino-8-naphthalenesulfonate.

Experimental Section

Materials. Sperm whale myoglobin was purchased from Seravac Chemical Co. (England). *Aplysia* myoglobin was prepared from the buccal muscles by the method of Rossi-Fanelli and Antonini (1957). Human hemoglobin was prepared by the toluene method (Drabkin, 1946).

The corresponding globins were obtained by acid acetone splitting according to Rossi-Fanelli *et al.* (1958a). The concentration of the globin was obtained spectrophotometrically using the following values for the molar extinction coefficients:

* From Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon (S. R. A.), and the Institute of Biochemistry and C.N.R. Center for Molecular Biology, University of Rome, and Regina Elena Institute for Cancer Research, Rome, Italy (M. B.), and the Biochemistry Division, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois (G. W.). *Received July 13, 1970.*
† Career Development Awardee 1 K04GM24134-01 of the National Institutes of Health. Address correspondence to this author or to M. B.

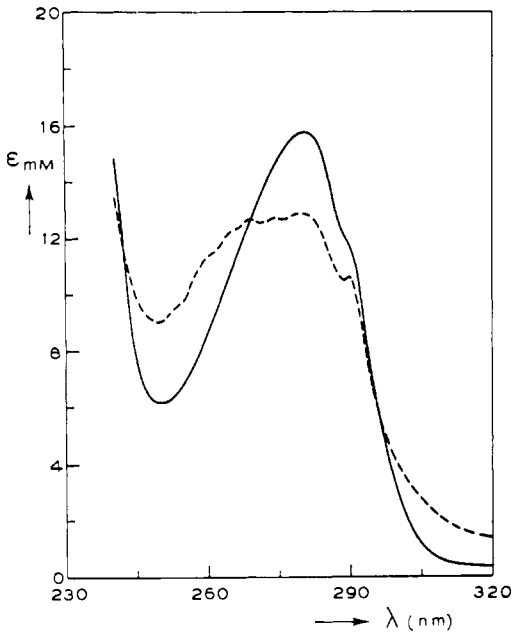

FIGURE 1: Absorption spectra of *Aplysia* apomyoglobin (– – –) and sperm whale apomyoglobin (——) at pH 7, 0.1 M phosphate, and 20°. The molecular weight is assumed to be 18,000 for each protein.

15,800 for sperm whale apomyoglobin at 280 nm; 13,000 for *Aplysia* apomyoglobin at 278 nm; 12,400 for human apohemoglobin at 280 nm.

The magnesium salt of 1-anilino-8-naphthalenesulfonate (ANS)[1] was prepared according to Weber and Young (1964). The fractional saturation of the protein with the dye was calculated from the known affinity constants (Stryer, 1965; Brunori *et al.*, 1968b). The conjugates of 1-dimethylamino-naphthalene-5-sulfonyl chloride (DNS) with the different apoproteins were prepared at neutral pH and 0° using a fivefold molar excess of the dye. The details of the labeling procedure were the same as reported by Anderson and Weber (1966). Each conjugate contained approximately 1 mole of DNS per mole of polypeptide chain.

All the experiments were performed in 0.1 M potassium phosphate buffer, pH 7. Most of the experiments were performed at a constant temperature of 15 ± 0.2°. Only the absorption spectra were measured at 20°. Performance of experiments at 20° or higher was avoided in view of the known relative instability of free globins under these conditions (Rossi-Fanelli *et al.*, 1958a).

Methods. Absorption spectra were determined with a Cary 15 recording spectrophotometer. Fluorescence emission and excitation spectra were measured using the fluorometer described by Weber and Young (1964). All the spectra were corrected for the wavelength dependence of the lamp output, grating transmission, and phototube response. Fluorescence polarizations were measured: (i) with a photoelectric fluorescence polarization photometer using a high-pressure mercury arc as light source (Weber, 1956). The 366-nm group of lines was isolated using Corning glass filters CS 7-60 and 4-71. The emitted light passed through liquid filters of 2 M $NaNo_2$ and Corning glass 3-72; (ii) with the instrument designed by Weber and Bablouzian (1966) for measurements in the ultraviolet region. This instrument was used to record fluorescence polarization spectra between 250 and 320 nm. The emitted light was filtered through Corning glass 0-52 which transmits no light at wavelengths shorter than 340 nm. The band widths of excitation were the same as those used by Anderson and Weber (1966).

[1] Abbreviations used are: ANS, 1-anilino-8-naphthalenesulfonate; DNS, 1-dimethylamino-5-naphthalenesulfonate.

TABLE I: Aromatic Amino Acid Content of Sperm Whale and Aplysia Myoglobins.[a]

	Sperm Whale	*Aplysia*
Try	2	2
Tyr	3	0
Phe	6	17

[a] Edmundson and Hirs (1961); Tentori *et al.* (1968, 1970, in preparation).

The fluorescence polarization (p) is related to the rotational relaxation time (ρ) of the kinetic unit carrying the emission oscillator and to the lifetime of the excited state (τ) by the Perrin equation

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + 3\frac{\tau}{\rho}\right) \qquad (1)$$

The limiting polarization (p_0) is the polarization obtained under conditions where depolarization due to Brownian motion is insignificant—*i.e.*, in rigid media of infinite viscosity (Weber, 1966).

The ratio ρ/τ can be determined experimentally by measuring the polarization as a function of T/η, the ratio of the absolute temperature to the viscosity of the solvent. A Perrin plot, constructed by plotting $1/p$ as a function of T/η, yields a straight line with $1/p = 1/p_0$ when $T/\eta = 0$; ρ/τ is given by

$$\frac{\rho}{\tau} = \frac{3\left(\frac{1}{p_0} - \frac{1}{3}\right)}{\left(\frac{1}{p} - \frac{1}{p_0}\right)} \qquad (2)$$

The viscosity of the solvent was adjusted to the desired value by the addition of solid sucrose.

The lifetime of the excited state was measured directly using the cross-correlation-phase fluorometer designed by Spencer and Weber (1969).

Results

1. Intrinsic Protein Fluorescence. Figure 1 shows the absorption spectra of *Aplysia* apomyoglobin and sperm whale apomyoglobin in the region between 240 and 320 nm. The two spectra reflect the differences in aromatic amino acid composition (see Table I). The fluorescence emission spectra obtained

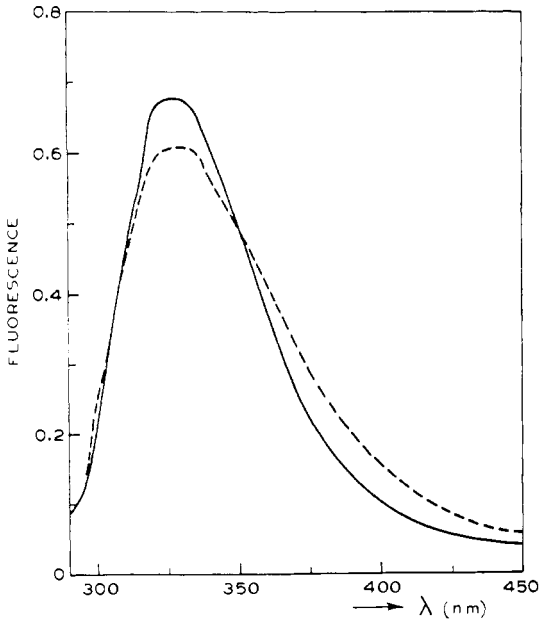

FIGURE 2: Emission spectra of *Aplysia* apomyoglobin (– – –) and sperm whale apomyoglobin (——). Conditions were: pH 7, 0.1 M phosphate, 15°; excitation, 288 nm; band width, 3.2 nm in both excitation and emission.

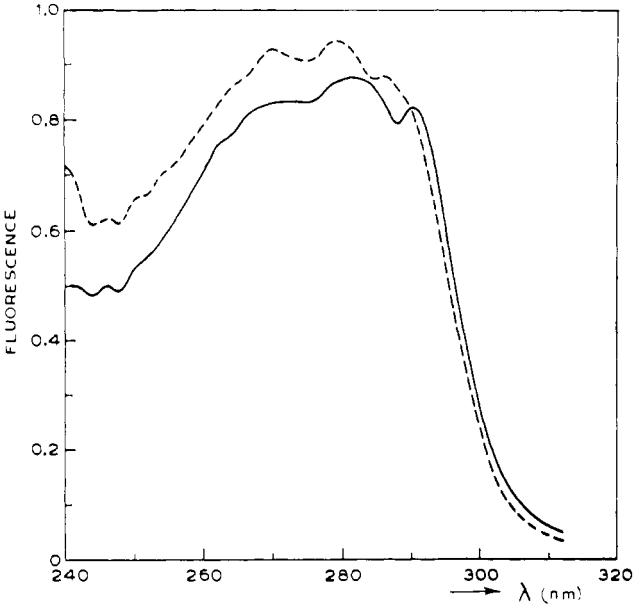

FIGURE 3: Excitation spectra of *Aplysia* apomyoglobin. Conditions were: pH 7, 0.1 M phosphate, 18°. Protein concentration was 6.5 $\times$ 10^{-5} M; observation at 307.5 nm (——) and 362 nm (– – –), which are isoemissive wavelengths; band width, 3.2 nm in both excitation and emission.

on excitation at 288 nm, where there is little absorption by either tyrosine or phenylalanine, are reported in Figure 2. Although both spectra have about the same emission maximum (λ_{max} 328–330 nm), the fluorescence spectrum of *Aplysia* apomyoglobin has a shoulder absent in the spectrum of sperm whale apomyoglobin. The position of this shoulder

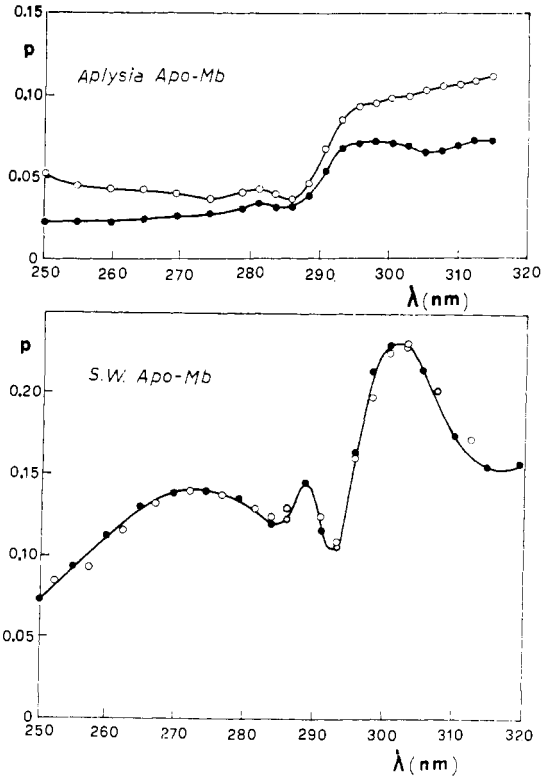

FIGURE 4: Fluorescence polarization spectra of sperm whale apomyoglobin (lower panel) and *Aplysia* apomyoglobin (upper panel). Conditions were: (●) proteins in buffer, pH 7; (○) the same in the presence of 29% sucrose. Protein concentration was 6.5 $\times$ 10^{-5} M in both cases, temperature, 12.5°.

(350–360 nm) corresponds to the emission maximum of tryptophan in aqueous solvents. This indicates that the tryptophan residues of the *Aplysia* protein are located in environments of different polarity. In accordance with this, the excitation spectra obtained at two isoemissive wavelengths (307.5 and 362 nm) are different in shape and magnitude (Figure 3). Comparison of these with the absorption spectrum (Figure 1) shows that the shoulder at 290 nm is considerably more pronounced in one of the two excitation spectra—*i.e.*, in that which should represent the chromophore in a nonpolar environment.

A relative quantum yield determination using free tryptophan in water at pH 7 and 17° as reference [q_{Try} = 0.20 according to Weber and Teale (1957)] indicates that q is approximately 0.12 in both apomyoglobins.

Figure 4 reports the fluorescence polarization excitation spectra of *Aplysia* apomyoglobin and sperm whale apomyoglobin recorded between 250 and 320 nm. In both cases the general qualitative features resemble those obtained for other proteins containing tryptophan (Weber, 1960). However there are obvious differences between the two apomyoglobins in the absolute values of the polarization and in the detailed shape of the polarization spectrum. In this respect the two apomyoglobins are as different as two completely unrelated proteins. The fluorescence polarization spectra were recorded before and after the addition of sucrose (29%) at a constant temperature of 12.5°. A considerable increase in polarization followed the addition of sucrose to *Aplysia* apomyoglobin

TABLE II: Fluorescence Properties of Apoheme Proteins.[a]

Chromophore	Apoprotein from	Maximum Emission (nm)	Fluorescence Yield, q	ρ/τ	τ (nsec)	ρ (nsec)	ρ/ρ_0	ρ/ρ_0'
Tryptophan	*Aplysia* myoglobin	330nm	0.12[c]	3.13	2.9 ± 0.1[d]	8.7	0.45	0.30
	Sperm whale myoglobin	328nm	0.12		2.8 ± 0.1[e]			
1,8-ANS	*Aplysia* myoglobin	478	0.90[f]	1.25	18.7 ± 0.3	23	1.2	0.79
	Sperm whale myoglobin	455	0.99	1.88	16.4 ± 0.4	31	1.6	0.17
	Human hemoglobin	455	0.99	3.65	16.7 ± 0.1	61		
DNS	*Aplysia* myoglobin	555		3.42	8.9 ± 0.2	30	1.55	1.04
	Sperm whale myoglobin	555	0.29[g]	2.72	11.0 ± 0.1	30	1.55	1.04
	Human hemoglobin	555	0.32		11.1 ± 0.1			

[a] Conditions employed were: pH 7.0; 0.1 M phosphate; 15°. [b] For myoglobin: ρ_0 = 19 nsec in H_2O at 15°; ρ_0' = 29 nsec. See text for calculations. [c] Values obtained by comparison to free tryptophan in aqueous solution (q = 0.20 at pH 7 and ~17°. Weber and Teale, 1957). [d] Value at 380 nm; at 340 nm, τ = 2.4 ± 0.1 nsec. [e] Value at 380 nm; at 340 nm. τ = 2.3 ± 0.1 nsec. [f] Values obtained by reference to bovine serum albumin–ANS complex (q = 0.70). [g] Values obtained by comparison to free DNS (q = 0.55).

while no appreciable change was found in the case of sperm whale apomyoglobin (Figure 4).

The polarization of fluorescence excited at a fixed wavelength (300 nm) was then determined isothermally at 15° as a function of solvent viscosity. The resulting Perrin plot (Figure 5) provides a value of ρ/τ for *Aplysia* apomyoglobin (Table II). Direct measurement of the lifetime of the excited state shows that this is the same in the two apoproteins—2.8–2.9 nsec at 380 nm. This value used in conjunctionw ith ρ/τ yields an apparent rotational relaxation time for *Aplysia* apomyoglobin of 8.7 nsec (Table II).

2. Fluorescence of the ANS Complex. Figure 6 shows the fluorescence emission spectra of the complexes of ANS with the two apomyoglobins. The emission maximum of the adsorbate of ANS with either sperm whale apohemoglobin or human apohemoglobin occurs at 455 nm and the quantum yield in both cases is nearly 1 (Stryer, 1965). The quantum yield of the complex of ANS with *Aplysia* apomyoglobin is lower (q = 0.90) and the emission maximum is shifted towards the red by 23 nm (λ_{max} 478 nm). The directly determined values of τ are identical for the ANS adsorbates with human apohemoglobin and sperm whale apomyoglobin, but somewhat higher for the complex with *Aplysia* apomyoglobin (Table II).[2]

The Perrin plots presented in Figure 7 and the directly determined lifetimes permit unequivocal determination of the rotational relaxation times (Table II). These relaxation times differ significantly from those calculated by Stryer (1965). If our experimental values of τ are used in conjunction with Stryer's values of ρ/τ at 20°, the values of ρ obtained are 69.8 nsec for ANS–apohemoglobin and 43.2 nsec for ANS–sperm whale apomyoglobin. For ANS–sperm whale apomyoglobin, there is an obvious discrepancy between our value of 31 nsec at 15° and Stryer's value of 43 nsec at 20°. No explanation is available to explain this difference.

3. Fluorescence of the DNS Conjugates. The fluorescence spectra of free DNS and of DNS conjugated with human apohemoglobin and with sperm whale apomyoglobin are shown in Figure 8. Interaction with the protein results in a decrease in the quantum yield to 50–60% of that of free DNS and in a red shift of the emission maximum ($\Delta\lambda_{max}$ 40 nm). The emission maximum is about the same in the three derivatives (Table II).

The limiting polarization obtained from Perrin plots is significantly different for sperm whale apomyoglobin (p_0 = 0.405–0.408) and for *Aplysia* apomyoglobin (p_0 = 0.385). The difference between the values of ρ/τ is entirely explained by a difference in τ. The rotational relaxation times of the two apomyoglobin conjugates are the same (30 nsec).

[2] When there is a competitive deactivation such as collisional quenching, the observed values of the lifetime and quantum yield are proportional. In the case of the two apomyoglobins, the ratio of the quantum yields of the ANS adsorbates is 0.91 while the ratio of the corresponding lifetimes is 1.12. This lack of proportionality can arise when there is an equilibrium between a fluorescent and a nonfluorescent form such as a complex of bound ANS with a solvent molecule.

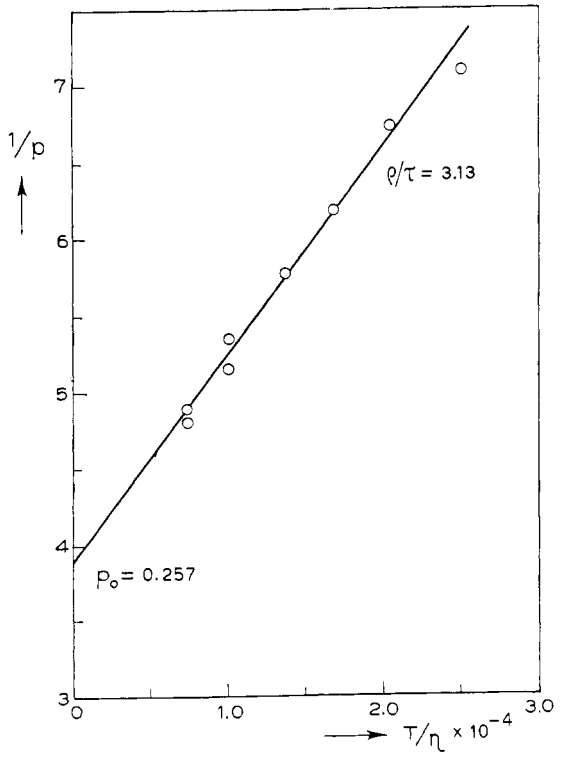

FIGURE 5: Perrin plot for *Aplysia* apomyoglobin (O). Conditions were: pH 7, 0.1 M phosphate, 15° (constant temperature). The viscosity was increased by addition of solid sucrose: protein concentration was 6.5×10^{-5} M; excitation, 300 nm; on the emission side: Corning 0–52.

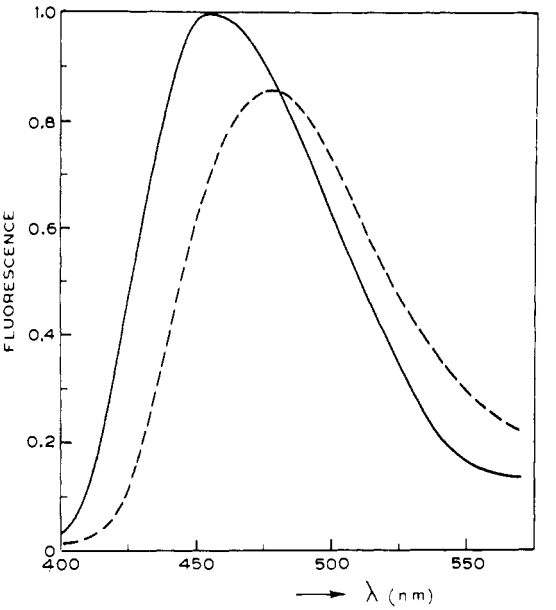

FIGURE 6: Emission spectra of the complexes of ANS with *Aplysia* apomyoglobin (– – –) and sperm whale apomyoglobin (——). Conditions were: pH 7, 0.1 M phosphate, 15°. Protein concentration was: 6.5×10^{-5} M: fractional saturation with ANS = 0.40; excitation at 360 nm; band width, 2.3 nm in both emission and excitation.

Discussion

Interaction with ANS. The binding of ANS by certain proteins results in a large increase in the fluorescence yield of the dye. The increase in quantum yield and the blue shift in the emission maximum have been correlated with the nonpolar character of the binding site (Weber and Laurence, 1954; Stryer, 1965). This correlation is particularly interesting in the case of adsorbates with apohemoproteins, where there is strong evidence that binding occurs at the same site where heme is normally bound (Stryer, 1965). The fluorescence properties of ANS bound to *Aplysia* apomyoglobin show that the specific dye binding site has nonpolar character. Comparison of the spectra and yields of the three adsorbates suggests that the hydrophobic character of the binding site in *Aplysia* apomyoglobin is less marked than in sperm whale apomyoglobin and in human apohemoglobin. However, we must remember that interaction of the excited ANS molecule with surrounding dipoles varies with mutual orientation and that dipole reorientation requires flexibility present in simple solvents but probably restrained in rigid macromolecules. Thus the spectra directly reveal the effectiveness of these interactions rather than the overall composition of the binding site (Brand, 1969).

Hydroaynamic Properties of the Globins. The conjugates of DNS with sperm whale and *Aplysia* apomyoglobins have identical rotational relaxation times (30 nsec) as well as similar emission and quantum yields (Table II). It is interesting to compare the relaxation time obtained from fluorescence polarization data with the value predicted for a sphere. The relaxation time of an anhydrous spherical molecule (ρ_0) is given by the expression $\rho_0 = (3\eta V/RT)$, where V is the molecular volume. Thus a molecule of molecular weight 18,000 and $\bar{V} = 0.74$ ml/g would have a value for ρ_0 of 19 nsec at 15°. The relaxation time of a hydrated sphere (ρ_0') is given by $\rho_0' = \rho_0(1 + h)$, where h is the hydration expressed as volume fraction of the anhydrous protein. Crystallographic and solution studies (Urnes, 1963; Kendrew, 1962) indicate that the hydration of sperm whale myoglobin is approximately 0.4 g of H_2O/g of protein ($h = 0.54$). Thus $\rho_0' = 29$ nsec for sperm whale myoglobin. The ratio ρ/ρ_0' reported in the last column of Table II is slightly higher than unity. This result can be easily explained by a small deviation from spherical symmetry since crystallographic data of sperm whale myoglobin indicate an axial ratio of 1.5 to 1.6 (Kendrew, 1962). Moreover the relaxation time obtained by Marcy and Wyman (1942) using dielectric dispersion on horse myoglobin is 31 nsec at 20°. We conclude that the overall hydrodynamic properties of sperm whale and *Aplysia* apomyoglobins are similar and that they are also similar to those of the corresponding myoglobins.

The rotational relaxation time of the ANS adsorbate of sperm whale apomyoglobin is the same as that obtained for the DNS conjugate. In *Aplysia* apomyoglobin, however, the relaxation time of the ANS adsorbate is significantly smaller (23 nsec). Since there are no hydrodynamic differences between apomyoglobin and its ANS adsorbate detectable in sedimentation measurements, this difference in ρ may reflect some localized flexibility in the *Aplysia* protein.

Rigorous interpretation of the rotational relaxation time of the complex of apohemoglobin with ANS is unrealistic in view of the lack of information on the hydrodynamic proper-

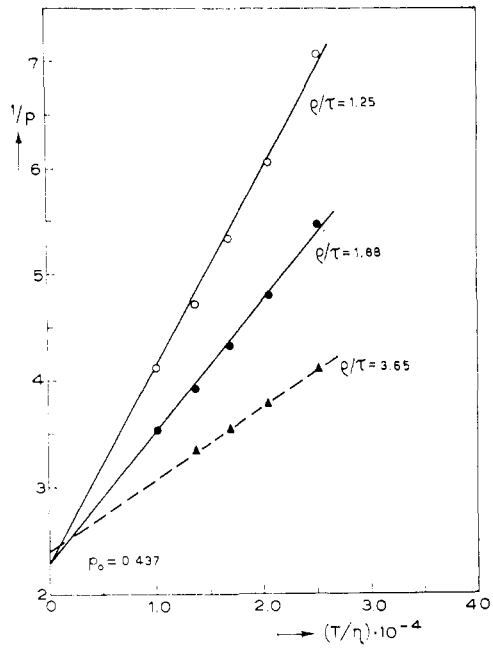

FIGURE 7: Perrin plots for the complex of ANS with the following apoproteins: (○) *Aplysia* apomyoglobin, protein concn 6.5 × 10^{-5} M saturation ~0.9; (●) sperm whale apomyoglobin, protein concn 4.2 × 10^{-5} M, saturation ~0.8; (▲) human apohemoglobin, protein concn 6.25 × 10^{-5} M; saturation ~0.3. Conditions were: pH 7, 0.1 M phosphate, 15° (constant temperature). The viscosity was increased by addition of solid sucrose: excitation, 366 nm. On the emission side: Corning 3-72 and liquid ultraviolet filter (2 M $NaNO_2$).

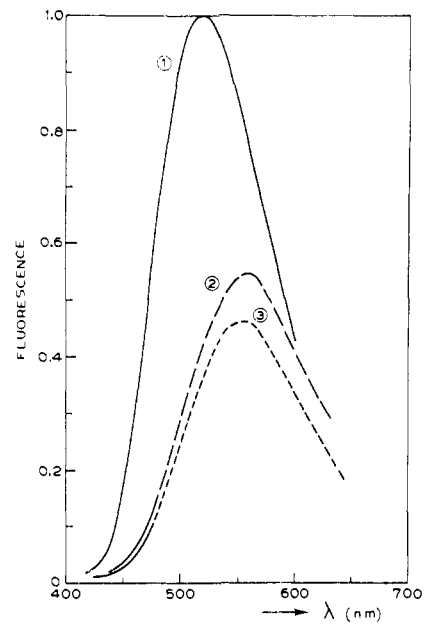

FIGURE 8: Emission spectra of free DNS (——) and of the conjugates of DNS with: human apohemoglobin (— — —), protein concn 3.7 × 10^{-5} M; sperm whale apomyoglobin (- - -), protein concn 1.7 × 10^{-5} M. Conditions were: pH 7, 0.01 M phosphate for free DNS and 0.1 M phosphate for the protein conjugates: 15°; excitation at 366 nm here the optical density was the same for the three samples (optical density = 0.184).

ties of the adsorbate. The fact that the relaxation time is approximately twice that of apomyoglobin is consistent with evidence that apohemoglobin is largely dimeric [mol wt = 41,000 according to Rossi-Fanelli *et al.* (1959)].

The study of the intrinsic fluorescence of the apoproteins from *Aplysia* and sperm whale myoglobins yields the most intriguing results. The fluorescence emission spectra reveal definite differences between the two proteins and in particular indicate that the tryptophan side chains of *Aplysia* apomyoglobin differ in their exposure to solvent. That is, one tryptophan is bound in a nonpolar region while the second is on the surface in contact with the water. This interpretation is confirmed by the excitation spectra obtained at two different emission wavelengths showing definite shifts in the absorption maxima (*e.g.*, 280–282 and 286–290 nm).

The fluorescence polarization spectra (Figure 4) reveal even more clearly the profound differences in the fluorescence properties of the two apomyoglobins and show that at any wavelength in the range examined the polarization of fluorescence of *Aplysia* apomyoglobin is significantly increased by the addition of sucrose. The isothermal Perrin plot (Figure 5) and the direct determination of the fluorescence lifetime yield an apparent relaxation time of 8.7 nsec for *Aplysia* apomyoglobin. This value is nearly 0.25 of that found in measurements on the DNS conjugate and must reflect local freedom of rotation. The details of the polarization spectrum and the low relaxation time of *Aplysia* apomyoglobin are characteristic of proteins in which the tryptophan residues are exposed to the solvent (Weber, 1960). Since the fluorescence was measured through Corning glass 0-52, the polar tryptophan will contribute more to the observed intensity than will the nonpolar tryptophan which emits primarily at wavelengths below the cutoff of our filter (340 nm).

A model consistent with all our observations on *Aplysia* apomyoglobin contains two different tryptophans: a nonpolar tryptophan side chain rigidly bound within the protein matrix and a mobile tryptophan side chain exposed to the solvent. In contrast, both tryptophan side chains of sperm whale apomyoglobin appear to be rigidly bound in nonpolar regions. The model for *Aplysia* apomyoglobin could be examined in a study of the relaxation times observed at different emission wavelengths. The exposure of the tryptophan side chains to the solvent could also be independently studied in measurements of the reactivity of the protein with reagents such as *N*-bromosuccinimide.

Acknowledgments

We express our warmest thanks to Mr. R. D. Spencer for direct measurements of the lifetime of the excited state. M. Brunori expresses his appreciation to the European Molecular Biology Organization for a short-term fellowship which made possible his visit to the University of Illinois.

References

Anderson, S., and Weber, G. (1966), *Arch. Biochem. Biophys. 116*, 207.

Brand, L. (1969), Johnson Foundation Colloquium on Probes of Molecular Structure held at University of Pennsylvania, Philadelphia, Pa.

Breslow, E., Beychok, S., Hardman, K. D., and Gurd, F. R. N. (1965), *J. Biol. Chem. 240*, 304.
Brunori, M., Antonini, E., Fasella, P., Wyman, J., and Rossi Fanelli, A. (1968a), *J. Mol. Biol. 34*, 497.
Brunori, M., Suelter, C., Kovacs, A. L., and Antonini, E. (1968b), Meeting of the Society of Italian Biochemists, Perugia, Italy, No. 290.
Daniel, E., and Weber, G. (1966), *Biochemistry 5*, 1893.
Drabkin, D. L. (1946), *J. Biol. Chem. 164*, 703.
Edmundson, A. B., and Hirs, C. H. W. (1961), *Nature (London) 190*, 663.
Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15*, 216.
Marcy, H. P., and Wyman, J. (1942), *J. Amer. Chem. Soc. 64*, 638.
Rossi-Fanelli, A., and Antonini, E. (1957), *Biokhimiya 22*, 335.
Rossi-Fanelli, A., and Antonini, E. (1960), *J. Biol. Chem. 325*, PC 4.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1958a), *Biochim. Biophys. Acta 30*, 608.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1959), *J. Biol. Chem. 234*, 2906.
Rossi-Fanelli, A., Antonini, E., and Povoledo, B. (1958b), *in* Symposium on Protein Structure, Neuberger, A., Ed., London, Methuen Co., p 144.
Spencer, R. D., and Weber, G. (1969), *Ann. N. Y. Acad. Sci. 158*, 361 (1969).
Stryer, L. (1965), *J. Mol. Biol. 13*, 482.
Tentori, L., Vivaldi, G., Carta, S., Antonini, E., and Brunori, M. (1968), *Nature (London) 219*, 487.
Urnes, P. J. (1963), Ph.D. Thesis, Harvard University, Cambridge, Mass.
Weber, G. (1956), *J. Opt. Soc. Amer. 46*, 962.
Weber, G. (1960) *Biochem. J. 75*, 345.
Weber, G. (1966), *in* Fluorescence Phosphorescence Analysis, Hercules, D. M., Ed., New York, N. Y., Wiley.
Weber, G., and Bablouzian, B. (1966), *J. Biol. Chem. 241*, 2558.
Weber, G., and Laurence, D. J. R. (1954), *Biochem. J. 56*, 31.
Weber, G., and Teale, P. W. J. (1957), *Trans. Faraday Soc. 53*, 646.
Weber, G., and Young, L. (1964), *J. Biol. Chem. 239*, 1415.
Wittenberg, B. A., Brunori, M., Antonini, E., Wittenberg, J. B., and Wyman, J. (1965), *Arch. Biochem. Biophys. 111*, 576.

Partial Modification of Bovine Serum Albumin with Dicarboxylic Anhydrides. Physical Properties of the Modified Species*

Ana Jonas† and Gregorio Weber

ABSTRACT: Bovine serum albumin was modified chemically with succinic, maleic, and citraconic anhydrides, and the properties of the derivatives were studied as to the nature and extent of the structural changes in the protein. Fluorescence polarization, rotational relaxation time, and sedimentation velocity measurements, as well as ionic strength, nature of salt, and pH dependence of fluorescence polarization experiments, led to the specification of conditions (*e.g.*, pH 7.0, water solution, 20°, for a 65% modified sample) under which the bovine serum albumin derivatives were shown to exist in an expanded form, very similar in its physical properties to bovine serum albumin expanded by electrostatic repulsion at pH 2. Modification of bovine serum albumin with citraconic anhydride was shown to be reversible; the citraconyl groups were easily removed under mild acidic conditions, without affecting the physical properties of the protein in relation to a control bovine serum albumin sample. The expansion of chemically modified serum albumin appeared to be a nonspecific process, depending on the gradual modification of about 80% exposed and 20% buried lysine residues in the protein.

Chemical modification with succinic anhydride was shown by Habeeb *et al.* (1958) to produce marked changes in the structure of bovine serum albumin. The conformational changes, detected by viscosity and sedimentation velocity measurements, were attributed to expansion resulting from the high charge density of the succinylated bovine serum albumin molecules. Similar results were obtained by Habeeb (1966), who determined the Stokes radii of chemically modified bovine serum albumin from elution volumes on Sephadex columns, and by Sun (1969), who measured the reduced viscosities of succinylated and partially succinylated bovine serum albumin as a function of pH.

In addition to bovine serum albumin many other proteins were reported to undergo structural changes on reaction with succinic anhydride, *e.g.*, γ-globulin and β-lactoglobulin (Habeeb *et al.*, 1958), transferrin, conalbumin, and orosomucoid (Bezkorovainy *et al.*, 1969), hemerythrin (Klotz and Keresztes-Nagy, 1963), and aldolase (Hass, 1964).

Butler *et al.* (1967, 1969) introduced maleic anhydride as a

* From the Department of Chemistry and Chemical Engineering, Biochemistry Division, University of Illinois, Urbana, Illinois 61801. *Received July 13, 1970.* This work was supported by a grant (U. S. Public Health Service Grant GM-11223) from the National Institutes of Health.

† Taken in part from the thesis submitted by Ana Jonas (U. S. Public Health Service predoctoral trainee) to the University of Illinois for the degree of Doctor of Philosophy in Chemistry. To whom correspondence should be addressed.