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Myoglobin as an Oxygen Indicator for Measuring the Oxygen Binding Characteristics of a Modified Myoglobin Derivative Containing Covalently Bound Mesoheme†

Philip D. Ross and Paul K. Warme*

ABSTRACT: By measuring the visible spectrum of a mixture of myoglobin and a modified derivative containing mesoheme in place of the normal protoheme, it is possible to evaluate the relative amounts of the oxidized, reduced, and oxygenated forms of each type of myoglobin. If the oxygen affinity of one myoglobin derivative is known, the oxygen affinity of the other can be determined from measurements at various oxygen partial pressures. In the absence of excess reducing agent, the rate of autoxidation can also be evaluated during the same experiment. The method described is suitable at very low oxygen partial pressures, where most previous methods are inaccurate, and it is very convenient to use, since no time-consuming calibration procedures are required. Using protoheme myoglobin as an oxygen indicator, the oxygen pressure at half saturation ($P_{1/2}$) of mesoheme myoglobin was shown to be 11% higher than the $P_{1/2}$ of a modified myoglobin derivative containing covalently bound mesoheme. The autoxidation rate of the covalent derivative is faster than that of the noncovalent derivative, but it is less dependent on oxygen pressure.

Mesoheme monosulfuric anhydride reacts specifically with lysine residue 45 in horse heart apomyoglobin to produce a modified myoglobin in which one of the heme propionic acid side chains is connected by an amide linkage to the lysine ϵ-amino group (Warme and Hager, 1970b). The visible spectrum of this covalent mesoheme myoglobin is almost identical with that of myoglobin containing noncovalently bound mesoheme, in both the oxidized and the reduced (oxy and deoxy) forms. In this paper, a novel equilibrium oxygen binding assay is utilized to demonstrate that the covalent mesoheme myoglobin derivative retains the ability to bind oxygen reversibly with an affinity very similar to that of noncovalent mesoheme myoglobin. The autoxidation rate of the covalent derivative is somewhat greater than that of the noncovalent derivative.

The extremely high oxygen affinity of myoglobin makes it quite difficult to measure its oxygen affinity accurately using current methods. This difficulty is reflected by the wide variation among reported values for the oxygen affinity of horse myoglobin, as measured by gasometric methods (Theorell, 1934; George and Stratmann, 1952), the tonometer method (Brunori et al., 1966), and the oxygen electrode method (Tamura et al., 1973a). Oshino et al. (1972) and Tamura et al. (1973a) have also used luminescent bacteria as an oxygen indicator for measuring the oxygen affinity of myoglobin derivatives.

As first shown by Hill (1939) and later by Davenport (1949), hemoglobin can be used as an indicator of oxygen concentrations in aqueous solutions. The fact that the visible absorption bands of mesoheme myoglobin are shifted about 10 nm to the red with respect to the absorption bands of normal (protoheme) myoglobin permits utilization of either of these proteins as an indicator of oxygen concentrations while measuring the oxygen affinity of the other. Thus, if the oxygen affinity of one derivative is known, the relative oxygen affinity of the other can be evaluated. Since each myoglobin derivative can exist in the oxidized state (met), the reduced state (deoxy), or the oxygenated state (oxy) and each of these forms has a distinct and characteristic absorption spectrum, the concentrations of each component of the equilibrium mixture can be determined by absorbance measurements at six different wavelengths.

Experimental Section

Materials

Horse heart myoglobin (type III), protoheme (type III), ferredoxin (type III), ferredoxin reductase, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XV), NADP, NADH, and catalase (type C-100) were all purchased from

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Sigma Chemical Co. Acrylamide, bisacrylamide and tetramethylethylenediamine (all electrophoresis grade) reagents were products of Eastman Chemical Co. DEAE[1]-cellulose (DE32) was obtained from Whatman and CM-Sephadex was a product of Pharmacia Fine Chemicals.

Methods

Preparation of Myoglobin Derivatives. Horse heart myoglobin was further purified by CM-Sephadex chromatography using the method of Hapner et al. (1968). Disc gel electrophoresis at pH 9.2 of the purified protein preparation gave a single band and confirmed the absence of a minor band which had been present in the unpurified myoglobin. Apomyoglobin and mesoheme were prepared as described earlier (Warme and Hager, 1970a,b) and the apoprotein was reconstituted with mesoheme by the procedure of Tamura et al. (1973b). The covalent mesoheme myoglobin derivative was prepared by reacting the mesoheme monosulfuric anhydride with apomyoglobin using the procedure of Warme and Hager (1970b). Both the covalent and the noncovalent mesoheme myoglobin derivatives were purified as recommended by Tamura et al. (1973b). About 40 mg of either protein was applied in 5 mM Tris-HCl buffer, pH 8.4, to a 1 × 12 cm column of DEAE-cellulose equilibrated with the same buffer. After washing the column with 6 mL of this buffer, a Tris buffer (5 mM, pH 8.4) containing 10 mM NaCl was used to elute the major heme protein band. The combined fractions were dialyzed against the initial Tris buffer and rechromatographed in the same manner. The single, symmetrical heme protein peak from this second column was dialyzed against water and concentrated on a Millipore Pellicon membrane, type PSACO.

Visible Absorption Spectra of Myoglobin Derivatives. The concentrations of myoglobin derivatives were routinely determined by the pyridine hemochrome method of Paul et al. (1953). The millimolar extinction coefficients at the α band were taken to be 31.0 for protoheme and 33.2 for mesoheme (Falk and Perrin, 1961).

The enzymatic reducing system of Hayashi et al. (1973) was used to prepare reduced myoglobin solutions. The following amounts of the various components were routinely used: 0.09 μmol of NADP, 1.33 μmol of glucose 6-phosphate (G6P), 0.67 nmol of G6P dehydrogenase, 0.35 nmol of ferredoxin, 0.16 nmol of ferredoxin reductase, and 0.0013 nmol of catalase.

In order to determine the extinction coefficients of the various states of each myoglobin derivative, all of the above reducing components except G6P dehydrogenase were dissolved in 1.98 mL of deoxygenated 0.1 M Tris-HCl buffer, pH 7.4, and sealed under argon in a small Thunberg cuvette containing a Teflon stir bar which had been deoxygenated under vacuum. A baseline spectrum was recorded using the computer-controlled spectrophotometer described below and then 0.02 mL of purified myoglobin solution (10 mg/mL) was added under argon. The spectrum of the metmyoglobin form was scanned before adding G6P dehydrogenase to initiate the reduction process. Addition of the dehydrogenase enzyme in the absence of myoglobin did not significantly affect the baseline. Argon was passed through the cell with constant stirring until no further reduction was reflected in the absorption spectrum and then the spectrum of deoxymyoglobin was recorded. After addition of 0.1 mL of oxygen-saturated Tris-HCl buffer, the oxymyoglobin spectrum was measured. The extinction coefficients obtained when sodium dithionite or methylviologen were used as the reducing agent agreed within 1% with those obtained using the enzymatic reducing system. The extinction coefficients used in the calculations were the average values from several experiments using all three reducing agents.

Oxygen Titrations of Myoglobin Derivatives. In titration experiments using methylviologen as the reducing agent, the baseline of deoxygenated Tris-HCl buffer (0.1 M, pH 7.4) was measured in a Thunberg cuvette sealed with a serum stopper and containing a deoxygenated Teflon stir bar. Purified myoglobin solution (10 μL) was added under argon, the metmyoglobin spectrum was scanned, and then portions of 15 mM reduced methylviologen in Tris buffer were added by hypodermic syringe until a faint blue color persisted. Since the reduced dye is rapidly oxidized to a colorless form in the presence of oxygen, its contribution to the absorbance is negligible under the conditions of subsequent measurements. Samples of air (20 μL) were added while stirring and scans were made after each addition. Titrations employing the enzymatic reducing system were done in a similar fashion, using G6P dehydrogenase to initiate reduction. After the myoglobin was completely reduced (about 10 min), spectral data were collected as the sample was further deoxygenated and subsequently oxygenated.

Specifications of the Computer-Controlled Spectrophotometer. A GCA-McPherson Model EU-707 double-beam ratio-recording spectrophotometer was modified as recommended (GCA-McPherson Co., technical communication) to scan in either wavelength direction under computer control. With this slight modification, spectral data could be collected automatically by a PDP-11 computer equipped with an RK05 1.2 million word disk and a Laboratory Peripheral System (LPS) containing an analogue voltage input module and a digital input/output module (all made by Digital Equipment Corp.). By sending the required number of pulses via the digital output channel, the monochromator was driven to a defined wavelength and the recorder output voltage (proportional to absorbance) was read into the computer via the analogue to digital converter of the LPS system. The accuracy of the absorbance data was verified using the potassium chromate standard recommended by Haupt (1952). In order to improve the accuracy of the absorbance values, the spectrophotometer output voltage was allowed to stabilize for 1 s before reading and averaging 100 consecutive voltage readings at each wavelength. The absorbance values were corrected by subtracting the stored data for the baseline. The maximum wavelength slewing rate permitted by this system is about 16 Å per s. The slit width was held at 250 μm for all measurements, providing a spectral band width of 5 Å. Due to slack in the chain drive which adjusts the angle of the monochromator grating, 15 extra pulses had to be delivered each time the direction of scanning was reversed. With this provision, a wavelength reproducibility of better than ±0.1 nm could be obtained between forward and reverse scans. A complete cycle, consisting of absorbance measurements at six different wavelengths in both the forward and reverse scan direction could be accomplished within 65 s. Absorbance changes in the samples were found to be linear within this short period, and, thus, the forward and reverse scan absorbance values were averaged to correct for changes in sample composition during the time required to measure the absorbance at different wavelengths.

Calculation of the Oxygen Binding Characteristics of Myoglobin Derivatives. A computer program was written in

[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl; G6P, glucose 6-phosphate.

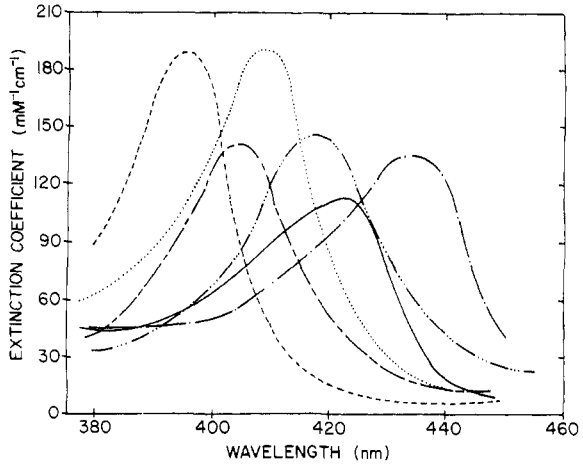

FIGURE 1: Comparison of the Soret spectra of protoheme and mesoheme myoglobin species at 5×10^{-6} M, 23 °C, 0.1 M Tris-HCl, pH 7.4. Mesoheme metmyoglobin (- - -), oxymyoglobin (- — -), deoxymyoglobin (—). Protoheme metmyoglobin (• • •), oxymyoglobin (• • •—• • •), deoxymyoglobin (•—•).

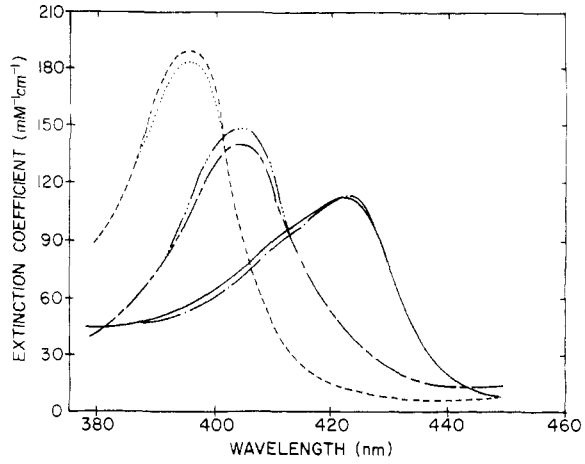

FIGURE 2: Comparison of the Soret spectra of covalent and noncovalent mesoheme myoglobin species at 5×10^{-6} M, 23 °C, 0.1 M Tris-HCl, pH 7.4. Noncovalent mesoheme metmyoglobin (- - -), oxymyoglobin (- — -), deoxymyoglobin (—). Covalent mesoheme metmyoglobin (• • •), oxymyoglobin (• • •—• • •), deoxymyoglobin (•—•).

Basic language to permit rapid collection of absorbance values as described above and to analyze these data to obtain oxygen binding parameters. In general, each type of myoglobin derivative can exist in three spectrally distinct states, so a mixture of two derivatives will contain six species. Absorbance values were determined at the absorbance maximum for each species for use in the following equation:

$$A^i = \sum_{j=1}^{6} \epsilon_j{}^i c_j \qquad (1)$$

where A^i is the total absorbance at wavelength i, $\epsilon_j{}^i$ is the extinction coefficient of species j at wavelength i, and c_j is the concentration of species j. The set of equations for six different wavelengths was solved by the method of elimination[2] to yield the concentrations of each of the six species present in solution under the prevailing experimental conditions. Of course, measurements at four wavelengths would suffice if the total concentrations (sum of all three species) of the two types of myoglobin derivatives are known, but the present method has the advantage that it does not require determination of these concentrations.

Knowing the concentration of each species in solution, the fraction of oxymyoglobin, Y_R, for each derivative, R, is determined by

$$Y_R = [\text{oxy}]_R/([\text{oxy}]_R + [\text{deoxy}]_R) \qquad (2)$$

The Hill equation (eq 3) permits calculation of the equilibrium constant, K_R, for binding of oxygen to myoglobin derivative R.

$$Y_R = \frac{K_R(P_{O_2})^n}{1 + K_R(P_{O_2})^n} \qquad (3)$$

By rearranging eq 3

$$n \log[P_{O_2}] = \log K_R + \log(Y_R/(1 - Y_R)) \qquad (4)$$

This equation must hold for each myoglobin derivative at equilibrium with oxygen so equations of this form for each of two types of myoglobin can be equated and rearranged to yield

$$\log K_2 = \log K_1 + \log(Y_1/(1 - Y_1)) - \log(Y_2/(1 - Y_2)) \qquad (5)$$

where the subscripts distinguish the two types. The oxygen pressure at half saturation, $P_{1/2}$, can be determined from eq 4 when $Y_R = 1 - Y_R$. Thus, K_R and $P_{1/2}$ for one type of myoglobin, and also the partial pressure of oxygen, can be evaluated from the experimentally determined concentrations of each species, provided that the K_R for the other type is known. We have used the $P_{1/2}$ value (1.03 mm) reported by Tamura et al. (1973a) for protoheme myoglobin as a reference standard because their luminescent bacteria method (Oshino et al., 1972) is most sensitive to oxygen levels in the concentration range of interest, and because they also employed the enzymatic reducing system, which ensures that no metmyoglobin is present during the measurements.

Results

The Soret band spectra of mesoheme myoglobin and protoheme myoglobin in their met, deoxy, and oxy states are compared in Figure 1 and similar comparisons of the covalent and noncovalent forms of mesoheme myoglobin are shown in Figure 2. These figures show that the spectra of the various forms of myoglobin are easily resolvable and that the spectrum of covalent mesoheme myoglobin is closely similar to that of the noncovalent mesoheme myoglobin. Our extinction coefficients at the peak positions agree closely with those given by Tamura et al. (1973a,b).

The success of our method is dependent on a certain degree of overlap between the oxygen binding curves of the two types of myoglobin being studied. At relatively high oxygen pressures, one or both types of myoglobin will be primarily in the oxy form, thus making the concentration of the deoxy form difficult to measure accurately. The converse will be true at low oxygen pressures. In order to establish the range of validity of our oxygen binding measurements, the $P_{1/2}$ values obtained from three different experiments on mixtures of mesoheme and protoheme myoglobin were plotted against log P_{O_2} and the average $P_{1/2}$ in the linear region was determined. The percentage difference between each individual $P_{1/2}$ value and the graphically determined average $P_{1/2}$ value was then plotted

[2] In order to reduce the amount of round-off error which accumulates during the calculations, the method of partial pivotal condensation was employed [Dorn, W. S., and McCracken, D. D. (1972), Numerical Methods with Fortran IV Case Studies, New York, N.Y., Wiley, pp 155-168].

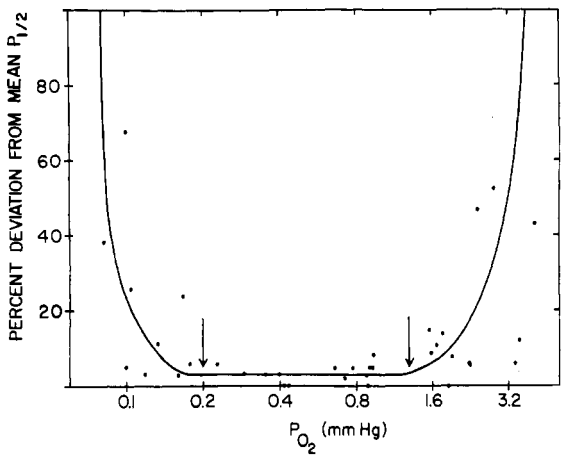

FIGURE 3: Relationship between error and oxygen levels in oxygen affinity measurements.

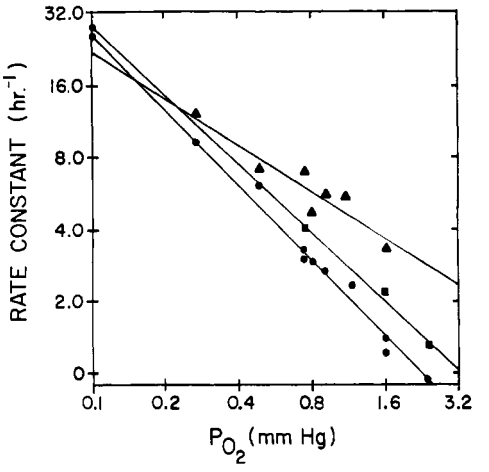

FIGURE 4: Dependence of the rate of autoxidation on oxygen partial pressure for protoheme myoglobin (●—●), noncovalent mesoheme myoglobin (■—■), and covalent mesoheme myoglobin (▲—▲).

against the calculated oxygen pressure for that measurement (Figure 3). As expected, the deviations from the mean are large at very high and very low oxygen pressures, but within a broad plateau region between 0.2 and 1.3 mm P_{O_2}, a small average deviation (3 to 7%) was observed.

At least three oxygen titrations were performed on each type of myoglobin using each of the two reducing methods, except for the case of covalent mesoheme myoglobin, for which only two titrations were done using methylviologen as reductant. Generally, five $P_{1/2}$ values were obtained from each titration, within the valid range of oxygen levels (0.2–1.3 mm). It was found that the average deviation among $P_{1/2}$ values within a particular titration experiment was less than 0.07 mm and that the average $P_{1/2}$ values obtained from independent titration experiments (using a particular type of myoglobin and reducing agent) varied by less than 0.05 mm. The measured values and literature $P_{1/2}$ values for the various myoglobin derivatives are summarized in Table I, along with the average deviation observed for each method. In all cases, Hill plots of log Y_R vs. the log of the partial pressure of oxygen (P_{O_2}) gave a linear plot with a slope of 1.0, thus confirming that the number of oxygen molecules bound per myoglobin molecule (n) is one, as expected. The calculated values of $P_{1/2}$ were found to be fairly insensitive to small errors in extinction coefficients. Although the extent of error varies with oxygen pressure and wavelength, the changes in $P_{1/2}$ obtained for a relatively large (1%) perturbation of a single extinction coefficient ranged from 0.0 to 0.08 mm.

When methylviologen is used as the reducing agent, some formation of metmyoglobin is unavoidable. Therefore, six simultaneous equations employing absorbance values for six wavelengths must be included in the calculations. However, no metmyglobin should form in the presence of the enzymatic reducing system, so that only four independent equations involving four different wavelengths are needed to solve for the unknown $P_{1/2}$ value. The fact that we obtained very similar results using data for either four or six wavelengths confirms that no metmyoglobin is present under enzymatic reducing conditions.

Using absorbance data for six wavelengths, it is possible to measure the rates of autoxidation of myoglobin derivatives as a function of oxygen pressure when methylviologen is used as the reducing agent. The rate constants at 15-mm oxygen pressure and the slopes of the log k vs. log P_{O_2} plots for various types of myoglobin are given in Table II and the autoxidation rates as a function of P_{O_2} are plotted in Figure 4. Since autoxidation rates are dependent on pH (Brown and Mebine, 1969), it is important to note that the values in Table II for bovine and sperm whale myoglobin were measured at pH 6.0 and the yellow fin tuna value is for pH 6.3. In general, the rate of autoxidation decreases with increasing pH, so the k values of the latter three types of myoglobin would be somewhat lower at pH 7.4, where our measurements on horse myoglobin derivatives were performed.

TABLE I: Oxygen Affinities of Horse Myoglobin and Derivatives Measured by Various Methods.[a]

Method	$P_{1/2}$ (mm O_2)		
	Protoheme	Noncovalent mesoheme	Covalent mesoheme
Gasometric	0.65[b]		
	1.16 ± 0.16[c]		
Tonometer	0.70[d]		
Oxygen electrode	1.2[e,f]		
Luminescent bacteria	1.03[e,f]	0.54[e,f]	
Oxygen indicator			
a. Methyl viologen reduction (six wavelengths)	(1.03)[g]	0.64 ± 0.02	0.58 ± 0.03
b. Enzymatic reductions (six wavelengths)	(1.03)[g]	0.70 ± 0.03	0.62 ± 0.04
c. Enzymatic reduction (four wavelengths)	(1.03)[g]	0.73 ± 0.02	0.65 ± 0.04

[a] All of our oxygen affinity measurements were performed in 0.1 M Tris buffer, pH 7.4, at 23 °C. Literature values were obtained under somewhat different conditions. [b] Theorell, 1934. [c] George and Stratmann, 1952. [d] Brunori et al., 1966. [e] Oshino et al., 1972. [f] Tamura et al., 1973b. [g] A standard value of $P_{1/2}$ = 1.03 for protoheme myoglobin was used in the calculations for the oxygen indicator method.

Discussion

Previously used methods for studying oxygen binding to myoglobin suffer from several sources of difficulty. The worst problem is that reduced myoglobin autoxidizes at significant rates when excess reducing agent is not present. This difficulty would affect measurements utilizing all methods cited in Table I, except the luminescent bacteria method and our method,

TABLE II: Autoxidation Parameters of Horse Myoglobin and Derivatives.[a]

	Slope of log k vs. log P_{O_2}	Value of k (h^{-1}) at 15 mmHg
Horse (protoheme)	−1.0	0.13
Horse (noncovalent mesoheme)	−0.94	0.25
Horse (covalent mesoheme)	−0.69	0.65
Bovine	−0.70[b]	0.44[b]
Yellow fin tuna	−0.34[b]	0.21[b]
Sperm whale	−0.68[b]	0.19[b]

[a] Conditions for our measurements were 0.1 M Tris buffer, pH 7.4, 23 °C. The literature values were obtained under somewhat different conditions. [b] Brown and Mebine, 1969. The pH was 6.0 for the bovine and sperm whale myoglobin measurements and pH 6.3 for yellow fin tuna myoglobin.

both of which use the enzyme reducing system. The rate of autoxidation is generally higher for modified myoglobin derivatives than it is for natural myoglobin, thus compounding this problem. In the presence of decomposition products of dithionite (and perhaps of other reducing agents as well), the rate of autoxidation is increased (Bembers et al., 1973). Our results demonstrate that the enzyme reducing system of Hayashi et al. (1973) is capable of reducing metmyoglobin as rapidly as it forms under experimental conditions.

The second major problem of some previous methods is that they lack sensitivity in the range of very low oxygen pressures required for study of the myoglobin binding equilibrium. For example, the signal-to-noise ratio of the Clark oxygen electrode is very low at oxygen pressures below 1.0 mm. On the other hand, the luminescent bacteria are very sensitive to low oxygen pressures but are not responsive above 1 mm P_{O_2}, so this method cannot be used to study oxygen binding equilibria of hemoglobin derivatives. In contrast, the method used in this paper is not dependent on the absolute oxygen pressure and only requires the availability of a suitable standard whose oxygen binding curve overlaps that of the unknown myoglobin derivative. Now that the oxygen affinity of both the covalent and noncovalent mesoheme derivatives of myoglobin have been determined accurately, they can be used as standards to measure the oxygen binding equilibria of normal (protoheme) myoglobin and its modified derivatives under various experimental conditions. Although we have not tested our method on hemoglobin, it is very likely that the deuterioheme or mesoheme analogue of hemoglobin would be an appropriate standard for measuring the oxygen affinity of various types of protoheme hemoglobin, since they have similar oxygen binding constants (Seybert et al., 1976). Of course, the mathematical treatment of the oxygen equilibrium in this case would require modifications to take into account any differences in the "n" values of the two proteins.

Other methods for measuring oxygen affinities of myoglobin derivatives typically require 5 to 30 times as much sample as our method requires. Since our measurements are made at the Soret band, where the extinction coefficients at the peaks are above 100 nM^{-1} cM^{-1}, less than 10 nmol of each myoglobin derivative are needed for measuring a complete oxygen binding curve and simultaneously measuring the rate of autoxidation. This advantage is significant when dealing with rare varieties of myoglobin or derivatives which are difficult to prepare in large amounts.

A further advantage of our equilibrium spectrophotometric method is that it avoids most of the tedious calibrations required by other methods which depend on the accurate response of some external system (e.g., an oxygen electrode or luminescent bacteria culture) to oxygen levels. Since both the standard and the unknown myoglobin are present in the same solution, the oxygen pressure and all other experimental conditions are necessarily identical for both proteins. Changes in sample volume are not important since only the proportions of the oxygenated and deoxygenated forms are used to calculate $P_{1/2}$. The fact that the total concentrations of the standard and unknown species are not required in the oxygen affinity calculations brings the added convenience that their absolute concentrations need not be determined by a separate measurement. Although our computerized spectrophotometer is a great convenience, the present method could be performed manually by scanning the Soret region after each addition of oxygen. In order to simplify the data acquisition, the enzyme reducing system is recommended, since it eliminates metmyoglobin and thereby eliminates two variables in the set of simultaneous equations.

The oxygen affinities reported in the bottom portion of Table I were highly reproducible within a given oxygen titration experiment and also agreed closely for multiple experiments on the same myoglobin derivative. The absolute values for $P_{1/2}$ are dependent on the standard value used for the reference compound, but, if a more accurate standard value is subsequently determined for protoheme myoglobin, our experimental values can be easily adjusted to conform by applying eq 5. The agreement between our $P_{1/2}$ value for mesoheme myoglobin and that obtained by the luminescent bacteria method is fairly close. Tamura et al. (1973a) do not specify the error interval associated with their measurements, so the significance of the observed difference is questionable. The small difference in $P_{1/2}$ values may be due to the fact that Tris buffer (0.1 M, pH 7.4) was used in our experiments, whereas phosphate buffer (0.1 M, pH 7.0) was used by Tamura and coworkers. Phosphate anion binds to myoglobin in a 1:1 molar ratio and apparently forms an electrostatic bridge between lysine-45 and the distal histidine residue 65 (Gillespie et al., 1966; Hartzell et al., 1968).

The $P_{1/2}$ values measured on samples reduced with methylviologen are lower than the corresponding values for samples reduced enzymatically. In the case of the covalent mesoheme derivative, this difference is within experimental error, but the difference is slightly outside the error estimate in the case of the noncovalent mesoheme derivative. An inherent assumption of our method is that the oxygen equilibrium is not affected by other components in the solution. Since all enzyme components of the enzymatic reducing system are present in amounts less than 1:18 relative to myoglobin, it is unlikely that the oxygen equilibrium is significantly perturbed by the other enzymes present. However, the ratios of NADP and G6P to myoglobin are about 0.75 and 110, respectively, so it is possible that these components could bind selectively to one or the other type of myoglobin and, thus, might affect the oxygen equilibrium. Methylviologen is readily autoxidizable, so that an excess must be added to accomplish complete reduction of myoglobin and any excess oxygen present in the solution. In our experiments, the ratio of methylviologen to myoglobin was typically about 50 to 1, and thus, it is possible that the oxygen equilibrium is affected by binding of methylviologen to the myoglobin derivatives. The fact that our experimental values using methylviologen agree as closely as they do with the $P_{1/2}$ values measured under enzymatic reducing conditions suggests that the perturbation is rather small.

As indicated by Figure 3, there is a wide latitude in the range of oxygen pressures suitable for relating the oxygen affinities of mesoheme and protoheme myoglobin derivatives. This permits multiple measurements of the oxygen affinity of the unknown derivative during an oxygen titration and thus provides increased accuracy of the averaged experimental results. This latitude in oxygen pressures also suggests that mesoheme myoglobin will be an excellent oxygen indicator for studying the oxygen binding equilibrium of protoheme myoglobin under varying experimental conditions.

The autoxidation rates of both mesoheme myoglobin derivatives are greater than that of protoheme myoglobin, as shown by Figure 4 and Table II. This finding is difficult to interpret, due to uncertainty regarding the mechanism of autoxidation. Although mesoheme is not the natural prosthetic group of myoglobin, it differs only in substituent positions 2 and 4, where ethyl groups replace the vinyl groups of protoheme. Apparently, this small structural difference destabilizes the molecule sufficiently to increase the rate of autoxidation. It is interesting to note that covalent mesoheme myoglobin has a slightly lower rate of autoxidation than either mesoheme or protoheme myoglobin at low oxygen pressures, whereas its rate is significantly higher than the others at high oxygen pressures. The sensitivity of the rates of autoxidation to small changes in the environment of the heme may prove to be useful for monitoring the effects of very slight modifications in myoglobin derivatives.

Our experimental results demonstrate that covalent attachment of mesoheme to myoglobin causes only a slight increase in its oxygen affinity. Because the heme group in this derivative is constrained to remain in close proximity to the polypeptide chain, it should confer added stability under denaturing conditions. Thus, denaturation studies on covalent and noncovalent mesoheme myoglobin may provide evidence concerning the role of the heme group in stabilizing the structures of heme proteins.

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