Oxygen and Carbon Monoxide Equilibria and the Kinetics of Oxygen Binding by the Cooperative Dimeric Hemoglobin of *Thyonella gemmata*†

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ABSTRACT: Oxygen and carbon monoxide equilibrium measurements have been made for the dimeric hemoglobin of *Thyonella gemmata*. For both ligands, the Hill number is ~1.4. The affinity for CO, however, is quite low, the median ligand activity at 20 °C, pH 7, being 1.3 μM. There is no apparent Bohr effect from pH 7 to pH 9. The replacement of O_2 by CO gives an apparent value for *M* of 2.4. More unusual is the finding that this replacement reaction is also cooperative, the apparent Hill number being 1.4, suggesting that, for this hemoglobin, the HbO_2 and HbCO conformations differ. HbO_2 – HbCO difference spectra in the 290-nm region suggest that replacement of O_2 by CO at the heme site is associated with the movement of a tryptophan from a nonpolar to a polar environment. The kinetics of oxygen dissociation from the hemoglobin of *T. gemmata* are biphasic. The dissociation rate constants for the hemes in the two polypeptide chains differ by a factor of about 5. Oxygen dissociation in the presence of CO is somewhat slower, but measurements at the HbO_2–HbCO isosbestic wavelength show that a maximum of nearly 1/2 of the hemes are unliganded during the reaction. This shows that the predominant half-liganded oxyhemoglobin species does not provide a quickly reacting form for CO binding, since the maximum CO association constant can be only ~2×10^{-4} M^{-1} s^{-1}. Oxygen-pulse experiments provide the clearest kinetic evidence for cooperativity in oxygen binding. The rate constants for oxygen dissociation from singly oxygenated forms must be greater than 5 times those for the fully oxygenated hemoglobin. The oxygen dissociation and oxygen-pulse measurements cannot be accommodated within a heterogeneous Adair model. In terms of an allosteric model, the rate constant for R → T or for T → R for intermediates such as $\alpha O_2 \cdot \beta$ is small ($t_{1/2}$ is tens of milliseconds), or the R ⇄ T equilibrium constant for the half-liganded form is ~1. The kinetics of oxygen association following HbCO photolysis are unusual in that the rate of oxygen binding is less than first power in oxygen. A model is proposed to account for these results. In this model, binding of oxygen to the initial photoproduct is some 30–40-fold slower than that to a second form, derived from the first by a conformational change, the $t_{1/2}$ for which is ~1 ms.

Hemoglobin was first reported in the echinoderms by Foettinger (1880) and in a holothurian by Howell (1885). Subsequently, hemoglobin has been reported in representatives of at least three of the five orders of holothurians (Terwilliger & Read, 1970, 1972; Manwell, 1959; Van Der Heyde, 1922; Hogben & Van Der Lingen, 1928; Hetzel, 1963). In all cases, the hemoglobin is found in hemocytes present both in the coelomic fluid and in the water-vascular system. Hemoglobins from the dendrochirotes *Cucumaria miniata*, *Cucumaria piperata*, and *Thyonella gemmata* are similar in amino acid composition, containing significantly larger amounts of isoleucine, aspartic acid, glutamic acid, and arginine and a smaller amount of histidine than does human hemoglobin. The number of amino acid residues per monomeric unit appears to be greater than that for either the α[1] or β chains of human hemoglobin and may be as large as 176 for *C. piperata* (Terwilliger & Read, 1970). The dimeric hemoglobins from the sea cucumbers *C. miniata* and *Molpadia oölitica* (Terwilliger & Read, 1970, 1972) appear to have two identical polypeptide chains yet show cooperative ligand binding. Chain heterogeneity has been reported for the hemoglobin from the sea cucumber *T. gemmata* (Manwell, 1966). Cooperative oxygen binding has been reported for *C. miniata* and *T. gemmata*; for both, the Hill number was 1.4 (Manwell, 1959, 1966). Owing to discussions concerning the role of the dimer in the cooperativity of human hemoglobin (Perutz, 1970; Gibson, 1970; Gibson & Parkhurst, 1968; Hewitt et al., 1972), there is considerable interest in the study of cooperativity in naturally occurring dimeric hemoglobins and myoglobins.

Oxygen binding kinetics make frequent use of CO as a competing ligand for oxygen dissociation studies or in order to prepare the protein in a highly photosensitive form (HbCO) for flash photolysis. Previous work (Parkhurst & Mobley, 1971) reported the unusually slow binding constant for CO, which suggested that the oxygen kinetics might differ from those frequently observed in that, for instance, oxygen dissociation in the presence of CO might yield a significant fraction of the hemoglobin in the unliganded state during the time course of the reaction. This was in fact observed and served to place an upper limit on the binding constant for CO to half-liganded oxyhemoglobin [see following paper, Parkhurst & Steinmeier (1979)].

Experimental Procedures

Materials

Sephadex G-25 and Sephadex G-100 were from Pharmacia Fine Chemicals, Inc., Piscataway, NJ. Carbon monoxide and argon, chemically pure, were from Matheson Co., Inc., East

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[1] The designation of chains as α and β does not imply a correspondence with mammalian hemoglobin chains. The α chain is arbitrarily assigned as that chain having the more rapid O_2 dissociation constant.

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Rutherford, NJ. Sodium dithionite, Manox Brand, was from Hardman and Holden, Ltd., Miles Platting, Manchester, England. Gel filtration marker proteins and sources were bovine serum albumin (Sigma Chemical Co., crystalline), chymotrypsinogen (Sigma Chemical Co., type 2), and sperm whale myoglobin (Calbiochem, A grade).

Live specimens of the sea cucumber *T. gemmata* were obtained from the Gulf Specimen Co., Panacea, FL. The average length was about 7 cm. The animals were bled by incising along the dorsal interambulacral space, from the anal end to a point near the base of the tentacular region. The coelomic fluid and the Polian vesicle fluid were collected in an equal volume of Alsever's solution (0.4% NaCl, 2% dextrose, and 0.03 M sodium citrate, pH 6.8). This material was filtered through glass wool and then centrifuged, at 4 °C, for 10 min at 10000*g*. The hemocytes were washed 3 times with 1% NaCl and then taken up in 5 volumes of 0.05 M potassium phosphate, pH 7. Hemolysis was achieved by three cycles of freeze-thawing, using an ice–salt bath at −8 °C. After lysis, the hemoglobin was separated from cell debris by centrifugation, at 4°, for 20 min at 27000*g*. Cellulose acetate electrophoresis at pH 8.6 in barbital buffer showed a main band, comprising more than 90% of the total hemoglobin, plus minor bands. Isoelectric focusing in acrylamide gels gave similar results. The hemoglobin was not purified further. Following fixation by 10% sulfosalicylic acid, staining was with 0.25% Coomassie Blue in 7.5% trichloroacetic acid and also with benzidine (1% in 75% acetic acid plus 0.3% H_2O_2).

Methods

Molecular weight determinations were made by gel filtration, using Sephadex G-100 columns, 0.9 × 80 cm (bed volume) and 0.9 × 40 cm. The buffer was 0.05 M potassium phosphate, pH 7. The marker proteins are listed above. For anaerobic conditions, the column was first equilibrated with oxygen-free pH 7 buffer to which sodium dithionite was added to a final concentration of 0.1%. Elution of markers and samples was carried out with the same buffer. Columns were monitored either at 280 or at 415 nm and volumes determined by counting drops (ISCO Model UA-2 monitor and drop detector, ISCO, Lincoln, NE). In the gel filtration experiments, the peak concentration on elution was 6–20 μM (80-cm column) and 5 μm (40-cm column).

Absorption spectra were taken with a Cary 14 spectrophotometer.

Oxygen equilibrium measurements were made by use of a 500-mL tonometer to which was fused a 5-mL 1-cm path length cuvette. Successive additions of air were injected through a serum stopper by using a greased syringe. The protein was equilibrated with the gas phase by gentle rotation for 10 min at 20 °C following each injection, and the absorption spectrum from 500 to 700 nm was then recorded. The methemoglobin reducing system of Asakura et al. (1972) was used for the equilibrium measurements. In the absence of the enzyme system, the deoxygenation process and subsequent manipulations resulted in substantial methemoglobin formation and loss of cooperativity.

Carbon monoxide equilibrium measurements were made in the following way. Concentrated stock oxyhemoglobin was diluted in a large glass syringe with argon-saturated buffer and then reduced by addition of small amounts of sodium dithionite. By mixing aliquots of this deoxyhemoglobin with solutions of known CO concentration (made by mixing solutions of CO-saturated and argon-saturated buffers), we prepared a series of solutions which contained different CO concentrations but maintained a constant heme concentration. Preparation of the solutions employed syringes calibrated by weighing. For the spectra of the individual samples, samples were transferred to a cuvette and covered with a thin layer of mineral oil to retard diffusion of the gases. Absorption spectra were recorded in the Soret region, and the data were processed so as to obtain a Hill plot.

Partition constant (M) measurements were made as follows. By mixing aliquots of a stock oxyhemoglobin solution with solutions containing known concentrations of CO and O_2, we prepared a series of solutions which contained different O_2/CO ratios but maintained a constant heme concentration. In all solutions, the total concentration of O_2 and CO was sufficient to maintain the hemoglobin fully liganded (greater than 99.9%). Spectra were recorded in the Soret region, and the data were processed so as to obtain a Hill plot.

Equilibration of buffers with gases, determination of gas concentrations, and a description of stopped-flow and flash-photolysis devices have all been reported previously (Steinmeier & Parkhurst, 1975). *Oxygen dissociation* kinetics were followed in the stopped-flow apparatus following rapid mixing of 10 μm (heme basis) *Thyonella* oxyhemoglobin in 0.05 M potassium phosphate buffer, pH 7, with the same buffer saturated with argon and containing 0.2% by weight dithionite at 20 °C. The observing wavelengths were 397, 415, and 440 nm. *Oxygen dissociation in the presence of CO* was carried out similarly to the above, except that the dithionite-containing buffer was saturated with CO so that, after mixture, the CO concentration was approximately 450 μM. Observing wavelengths were 397 and 414 nm. At 397 nm, Hb and HbCO are isosbestic; at 414 nm, HbO_2 and HbCO are isosbestic.

Oxygen-pulse measurements were similar to those of Gibson (1973). In these experiments, deoxyhemoglobin solutions containing dithionite were flowed against solutions of varying oxygen concentration. In this manner, contributions to the dissociation kinetics from partially oxygenated intermediates could be observed. Sodium dithionite stock solutions were 0.2% (w/v), prepared by dissolving a weighed amount of $Na_2S_2O_4 \cdot 2H_2O$ in the appropriate volume of argon-saturated 0.05 M potassium phosphate pH 7 buffer. Stock oxyhemoglobin solutions were 575 μM in heme. For calibration purposes, a 575 μM whale metmyoglobin solution was also prepared. For kinetic experiments, these stock solutions were further diluted in oxygen-free pH 7 buffer. Final solutions contained 10 μM hemoglobin or 10 μM myoglobin plus either 0.01% or 0.05% dithionite. Solutions of known oxygen concentration employed in the kinetic measurements were prepared from air-equilibrated pH 7 phosphate buffer. The oxygen concentration of the latter was obtained by calculation, based on the ambient temperature, atmospheric pressure, average composition of air, and the aqueous solubility of oxygen (Hodgman, 1962). The air-equilibrated buffer was diluted by using argon-saturated pH 7 buffer. When hemoglobin and myoglobin results were to be compared directly, experiments for both heme proteins were performed by using the same oxygen solutions. The hemoglobin concentration was 10 μM before mixing (heme basis), and the oxygen concentrations ranged from 264 to 33 μM before mixing. Measurements were made at 20 °C and were observed at 440 nm.

Oxygen association kinetics were followed by monitoring absorbance changes following flash photolysis of HbCO in the presence of oxygen. The concentration of hemoglobin varied from 5 to 10 μM, that of CO was usually 750 μM, and the oxygen concentration varied from 50 to 200 μM. The buffer was 0.05 M potassium phosphate, pH 7. The temperature was

Table I: Ligand Binding Equilibrium Data for *T. gemmata* Hemoglobin[a]

ligand	$\overline{X}$	n
O_2	5.2 ± 2 μM	1.38 ± 0.04
CO	1.3 ± 0.2 μM	1.40 ± 0.08
CO/O_2 (M determination)	$M = 2.4$	1.4 ± 0.1

[a] Conditions were as follows: buffer, 0.05 M potassium phosphate, pH 7; $T = 20$ °C. Protein concentrations: for O_2 equilibrium, 10 and 60 μM in heme; for CO, 5 μM; for M, 5 μM. $\overline{X}$ is the half-saturation ligand activity; n is the Hill number.

20 °C and the observing wavelength 427 nm.

Data collection and reduction methods have been described previously as well as methods of fitting the data to one- and two-exponential models (Steinmeier & Parkhurst, 1975). Numerical integrations of the oxygen-pulse mechanisms were by fourth order Runge–Kutta methods, and the fitting of the oxygen association data according to various models was by direct grid search similar to that employed by Becsey et al. (1968). The latter two calculations were carried out on a Super-Nova computer, Data General Corp., with 20K of core memory.

Results

Small-zone gel filtration experiments gave a molecular weight of 33 000 for oxyhemoglobin, in agreement with the value 35 000 previously reported (Parkhurst & Mobley, 1971). The results were independent of column length and protein concentration for the conditions given above. The deoxyhemoglobin data yielded a molecular weight of 32 000. These results suggest the absence of detectable differential (oxy vs. deoxy) aggregation for *Thyonella* hemoglobin in the concentration region of 10 μM in heme. We had previously shown (Parkhurst & Mobley, 1971) that in $NaDodSO_4$–acrylamide gel electrophoresis, the *Thyonella* monomer migrates just behind myoglobin. On the basis of amino acid analysis of the protein, the calculated subunit molecular weight is 17 500. Figure 1 shows a Hill plot of the oxygen equilibrium results at pH 7 for two concentrations of hemoglobin, 10 and 60 μM in heme. No concentration dependence was evident. Linear least-squares analysis yielded the values in Table I. No alkaline Bohr effect was evident for *Thyonella* hemoglobin since pH 9 data were identical with those obtained at pH 7. A Hill plot for CO equilibrium for hemoglobin 5 μM in heme gave a value for the Hill number of 1.4, essentially the same as that obtained for oxygen binding. The CO affinity is strikingly low, however, the median ligand activity being 1.3 μM. The partition coefficient, M, for HbO_2–HbCO equilibration is usually defined in terms of the overall reaction

$$HbO_2 + CO \rightleftarrows HbCO + O_2$$

$$M = \frac{(HbCO)(O_2)}{(HbO_2)(CO)} = K/L$$

where K and L are respectively the dissociation equilibrium constants for oxygen and CO binding. For human hemoglobin, data fit the above equation, giving the same value of M for any ratio of HbO_2 to HbCO (Antonini & Brunori, 1971). At 20 °C and pH 7, M for human hemoglobin is about 200. For *Thyonella* hemoglobin, however, it was found that M did in fact vary with the ratio of HbO_2 to HbCO. Experimental results were treated by graphing the log of $(HbCO)/(HbO_2)$ vs. the log of $(CO)/(O_2)$ as in a Hill plot. The Hill number for this plot was 1.4. Thus, for *Thyonella* hemoglobin, the replacement reaction of O_2 by CO appears to be a cooperative process. For the mixture containing half HbO_2 and half HbCO, the apparent value of M is 2.4. The value of n, the Hill number, for this process suggests that the HbO_2 and HbCO conformations may differ significantly.

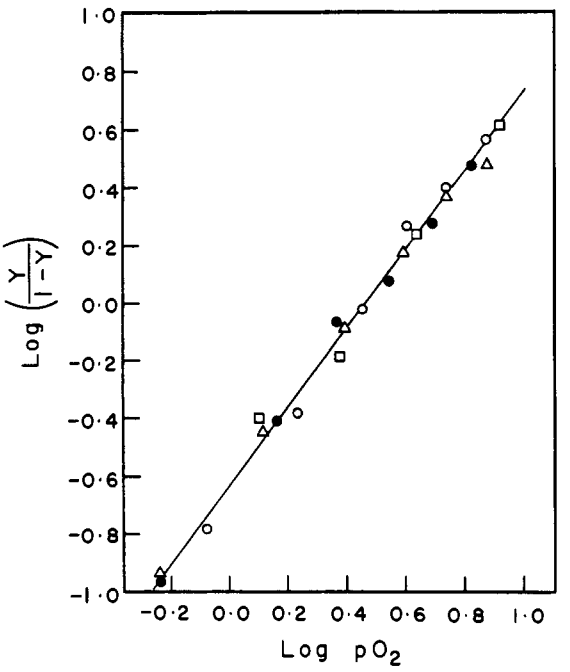
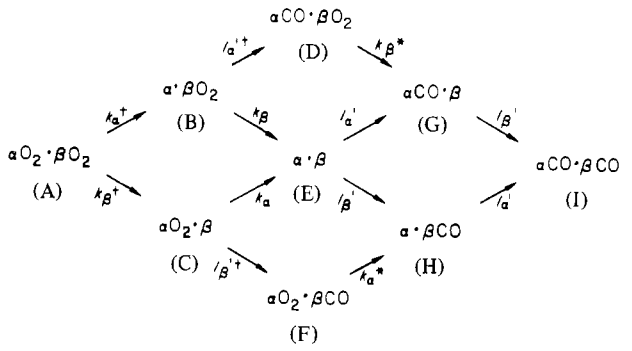


FIGURE 1: Hill plot of oxygen equilibrium data for *Thyonella* hemoglobin. Measurements were at pH 7 in 0.05 M potassium phosphate buffer at 20 °C. Different symbols refer to separate determinations. Filled symbols are for protein concentration 10 μM in heme, and open symbols are for 60 μM in heme; oxygen pressure in millimeters of mercury.

Difference spectra for human and *Thyonella* hemoglobins in the region of 280 nm lend support to the suggestion of the conformational differences between the oxy and CO forms of *Thyonella* hemoglobin. Absorption spectra for the oxy and CO forms for the two hemoglobins (human and *Thyonella*) differ in peak position and isosbestic points by at most 2 nm, for the Soret and α–β-peak regions. In contrast, comparison of spectra in the ultraviolet showed marked difference between the two hemoglobins. Figure 2 shows HbCO – HbO_2 difference spectra for human and *Thyonella* hemoglobins. The human difference spectrum has been attributed to contributions from heme absorptions (Briehl & Hobbs, 1970). The broad bands shown here for *Thyonella* hemoglobin undoubtedly arise in part from ligand-dependent heme absorption in this region; however, for human hemoglobin, the HbCO–HbO_2 isosbestic point is 284 nm; for *Thyonella*, it is 277 nm, which, on an energy basis, is more than 10 times the difference found in the visible region for the isosbestic points. Reasoning that the heme contributions in the 280-nm region should be similar owing to similarities elsewhere, we constructed the difference of the two difference spectra (Figure 2, dotted curve). If the heme contributions canceled, then this spectrum should reveal changes in the protein moiety of *Thyonella* hemoglobin when O_2 is replaced by CO, assuming no changes occur in human hemoglobin.

Oxygen dissociation kinetics in the absence of CO displayed biphasic behavior. The rapid component comprised 46% of the total signal at 415 and 440 nm. The rate constants were 18 and 3.6 s^{-1}. Oxygen dissociation kinetics in the presence of CO were considerably more complex. In terms of a heterogeneous Adair mechanism, we can describe the reaction by



In this scheme, rate constants relating to the oxy conformation are denoted by (†) and those relating to the $HbCO{\cdot}O_2$ conformation by (*). Rate constants for oxygen dissociation from half-oxygenated species are also distinguished from the other two classes. CO association constants are also distinguished according to whether combination is to a site, the partner of which is occupied by oxygen or is empty. In the scheme above, the l' is the pseudo-first-order rate constant. There is no evidence (see following paper) that CO rate constants differ for binding to a form such as (E) or to (H) and thus are not distinguished above. The differential equations for this simple network are readily solved by LaPlace transforms and the inversions readily found from the residues of the Bromwich integral. The general absorbance change for this network has eight exponential decay terms, the decay constants being $k_\alpha^\dagger + k_\beta^\dagger$, $k_\beta + l_\alpha'^\dagger$, $k_\alpha + l_\beta'^\dagger$, $l_\alpha' + l_\beta'$, k_α^*, k_β^*, l_α', l_β'. It is, of course, experimentally impossible to obtain so many constants from these experiments. The problem is simplified somewhat by judicious choices of wavelengths. For instance, at 397 nm, the Hb–HbCO forms are isosbestic; at 414 nm, the HbO_2–HbCO forms are isosbestic. In the former case, absorbance-change measurements can be described in terms of only the five species A, B, C, D, and F above, and the general absorbance change would have only five decay constants (the first three above plus k_α^* and k_β^*), which is, however, still too large a number to be determined from the data. Observations at 414 nm can be described in terms of only the five species B, C, E, G, and H but can be described in terms of all eight decay constants. If, however, one assumes that all k's are the same, and all l's the same, aside from α–β heterogeneity, then the normalized absorbance change at 397 nm is simply $[\exp(-k_\alpha t) + \exp(-k_\beta t)]/2$. The experimental curve is biphasic with rate constants 15 and 3 s^{-1}, 20% slower than when CO was absent, suggesting, as might be expected, that loss of oxygen from the saturated oxyhemoglobin is slower than the overall rate of oxygen release. Usually, the oxygen dissociation experiment in the presence of CO permits one to obtain k_α^* and k_β^* since the reaction proceeds essentially from A to D and F, and thence to I, with no detectable unliganded intermediates. This requires that the CO association rate constant for half-liganded forms, or for tetrameric hemoglobins, tri-liganded forms, be much greater than the oxygen dissociation constant from the liganded conformation. Were this the case here, then observations at 414 nm would show no absorbance change. In fact, studies at 414 nm show a large transient absorbance change corresponding to 47% unliganded hemoglobin at the maximum, showing that species such as B, C, E, G, and H are significant in this reaction. If we set all k's equal and all l's equal, it can be shown that the transient absorbance change at 414 nm for the above scheme would correspond to the appearance of the intermediate B in a scheme

$$A \xrightarrow{k} B \xrightarrow{l'(CO)} C$$

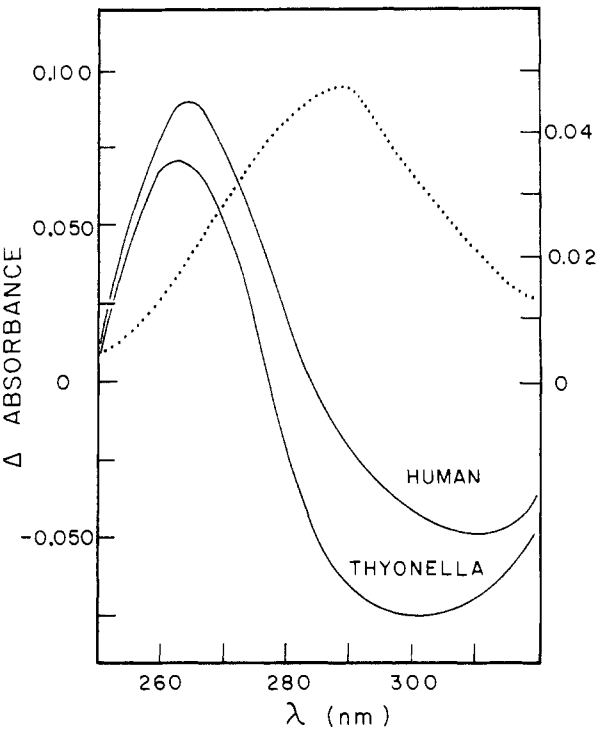


FIGURE 2: HbCO minus HbO_2 absorption spectra. Upper curve, human hemoglobin; lower curve, *Thyonella* hemoglobin. The dotted curve was produced by subtracting the *Thyonella* difference spectrum from that for human hemoglobin. Spectra were for hemoglobins 20 μM in heme in 0.05 M potassium phosphate buffer, pH 7, at 20 °C.

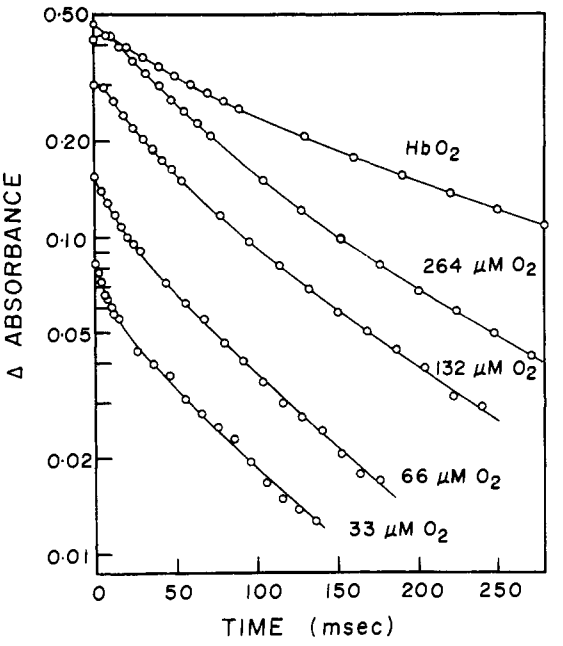


FIGURE 3: Oxygen-pulse experiments on *T. gemmata* hemoglobin. The top curve is for deoxygenation in which oxyhemoglobin is mixed with dithionite. Indicated oxygen concentrations are before mixing in the stopped-flow apparatus. Experiments used 10 μM hemoglobin (heme basis) and 0.05% dithionite (before mixing). Measurements were at 20 °C in 0.05 M potassium phosphate, pH 7, observed at 440 nm.

If we let $X = l'(CO)/k$, $\log [(B)/A_0]_{max} = [X/(1 - X)] \log X$, and, for 50% unliganded hemoglobin as the intermediate, $X = 1/2$, or $l' = 2 \times 10^{-4}$ M^{-1} s^{-1}, showing that there is no quickly reacting form of *Thyonella* hemoglobin with respect to CO that is detectable in this experiment. Figure 3 shows the results of the oxygen-pulse experiments. The maximum

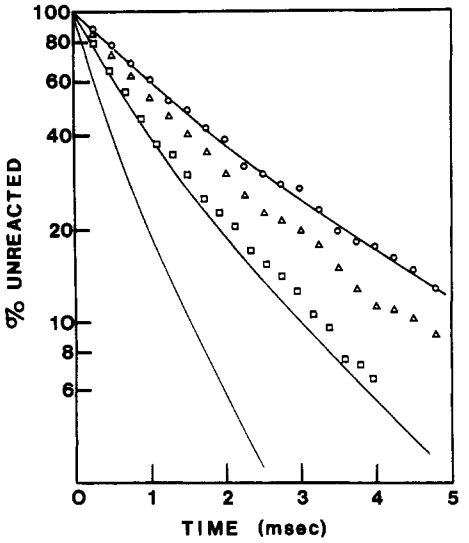


FIGURE 4: Kinetic curves for oxygen binding obtained from flash photolysis of *Thyonella* HbCO in the presence of known concentrations of oxygen. Measurements employed 5 μM hemoglobin in 0.05 M phosphate, pH 7, at 20 °C and were observed at 427 nm. Data points represent experiments at the oxygen concentrations 51.5 (○), 103 (△), and 200 μM (□). In a given experiment, the total CO concentration was not less than 750 μM. The top solid curve is the best two-exponential fit to the 51.5 μM oxygen data; the remaining curves are those which would be obtained were the oxygen binding rate first power in oxygen.

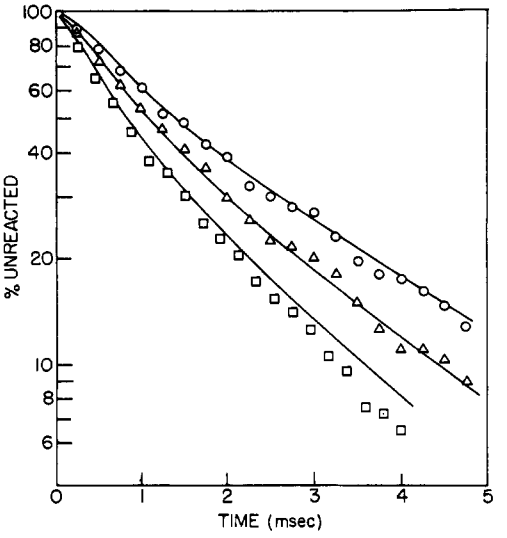


FIGURE 5: Computer fits of oxygen binding kinetics to a branching mechanism. Data points are those of Figure 2 and represent three of the ten curves used in the fitting. Parameter values are the final values from Table II.

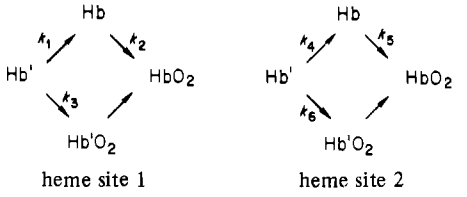
absorbance change for each curve reflects the extent to which oxygenation of heme sites occurs for that reaction. Thus, for the bottom curve, at a maximum 17.5% oxygenation, the initial rate for oxygen dissociation was slightly in excess of 100 s^{-1}, more than 5 times that of the rapid component observed in the usual deoxygenation kinetics.

Oxygen association was studied by flash photolysis of carboxyhemoglobin in the presence of known concentrations of oxygen. Photodissociation of the CO is followed by a rapid combination reaction with oxygen. For *Thyonella* hemoglobin, the CO recombination reaction is more than 1000 times slower and does not interfere. Typical experimental results are shown in Figure 4. The O_2 binding reaction occurred rapidly and was essentially complete within 10 ms for the oxygen concentrations employed. The apparent overall rate constant was $\sim 10^7$ M^{-1} s^{-1}. Remarkably, however, successive twofold increases in oxygen concentration gave only about a 50% increase in the overall rate of O_2 combination. Thus, the solid lines in Figure 4 are the curves expected for the two lower oxygen concentrations, based on the top curve for 200 μM oxygen. Furthermore, the experimental curves were biphasic, decelerating as the reaction approached completion. These unusual results may well derive from the apparent conformational differences between HbCO and HbO_2 for *Thyonella* hemoglobin. A mechanism which gives reasonable fits to the data is shown below. This branching mechanism assumes that the hemoglobin present after photolysis (Hb′, which represents a CO-bound protein conformation) either reacts directly with O_2 or undergoes a conformational change to another form, perhaps the usual deoxy conformation, Hb, which then reacts with O_2. The conversion of $Hb'O_2$ to HbO_2 is assumed to occur without detectable absorbance change. Analysis of the experimental data in terms of the branching mechanism employed a direct grid search program. Results of the computer fitting are shown in Figure 5, the data points being those from Figure 4. Although only three curves are shown, the computer fitting utilized a set of ten experimental curves at six different O_2 concentrations. Initial parameter estimates and final values are shown in Table II. Parameter variation covered a range 50 times higher and 50 times lower than the initial estimates. A total of 187 500 points in the six-dimensional parameter space were examined in finding the optimum fitting parameters for the 10 curves, 200 data points, at 6 oxygen concentrations. The branching mechanism was

Table II: Initial and Final Parameters for the Branching Oxygen Association Mechanism[a]

rate constant	initial value	final value
k_1	600 s^{-1}	1600 s^{-1}
k_2	1.0×10^7 M^{-1} s^{-1}	6×10^7 M^{-1} s^{-1}
k_3	5×10^6 M^{-1} s^{-1}	1.5×10^6 M^{-1} s^{-1}
k_4	550 s^{-1}	400 s^{-1}
k_5	3.5×10^6 M^{-1} s^{-1}	1.3×10^7 M^{-1} s^{-1}
k_6	1.75×10^6 M^{-1} s^{-1}	4.5×10^5 M^{-1} s^{-1}

[a] A total of 187 500 points were searched in the six-dimensional parameter space. Ten curves, each with twenty data points, were fit. Each curve was normalized to an initial value of 1. The final sum of squared residuals for the 200 data points, 10 normalized curves, was 0.055, which was within experimental error.



Discussion

The results of the equilibrium measurements show that the dimeric hemoglobin is cooperative for ligand binding both with respect to CO and oxygen. The unusual results are the low affinity for CO (and the correspondingly low value for M) and the apparent nonequivalence of the conformations of HbO_2 and HbCO. Evidence for this nonequivalence derives from the M determinations and difference spectra reported here, differences in the 280-nm region in the CD spectra (Geraci et al., 1972), and peculiarities in the oxygen combination kinetics. With regard to Figure 2, dotted curve, it is known that transfer of protein tryptophan residues from a nonpolar to a polar environment produces a difference spectrum with a maximum at 292 nm. At that wavelength the average decrease in molar absorptivity is 1600 (Donovan, 1969). For

tyrosine residues, a maximum is found at 287 nm, and the decrease in the molar absorptivity is 700. In *Thyonella* hemoglobin, changing the ligand from O_2 to CO apparently results in increased exposure of aromatic chromophores to the solvent. Under the conditions used for the experiments shown in Figure 2, exposure to the solvent of one tryptophan per heme should alter the protein absorbance at 292 nm by 0.032. Exposure of one tyrosine per heme should alter the absorbance at 287 nm by 0.014. Since there are only three tyrosine residues per monomer, it appears that tryptophan is very likely involved in a conformational change when CO replaces O_2 in *Thyonella* hemoglobin. A somewhat related gross change in properties was found in *Lumbricus* hemoglobin, where the kinetics of protein dissociation were markedly affected by the replacement of O_2 by CO (Goss et al., 1975).

The kinetics of oxygen dissociation from *Thyonella* hemoglobin are biphasic with apparent rate constants of 18 and 3.6 s^{-1} at 20 °C, pH 7. When the oxygen dissociation reactions are studied by oxygen-pulse measurements, at low fraction initial oxygenation the reaction is also biphasic, but now the two apparent rate constants are $\sim$100 and $\sim$12 s^{-1}. This latter experiment provides the clearest kinetic evidence of cooperativity, showing that the predominant oxygenated forms at low fractional saturation lose oxygen at least 5 times more rapidly than does the fully liganded form. These experiments also show that the hemes in the two polypeptide chains are kinetically distinct. If we were to use an Adair model to describe oxygen dissociation, then, in terms of the above diagram, the species A–C and E would be involved. If we assign rate constants greater than 100 and 12 s^{-1} to the latter two rate constants, k_α and k_β, then we must be able to describe oxygen dissociation in terms of two additional constants, $k_\alpha^\dagger$ and $k_\beta^\dagger$, such that the apparent rate constants are 18 and 3.6 s^{-1} and the fraction rapid is 47%. An extensive investigation of this problem, using the Powell algorithm (Powell, 1965), showed that there were no satisfactory real, nonnegative rate constants for such a kinetic model. The evidence then favors a more elaborate model, such as a heterogeneous allosteric model [see Parkhurst & Steinmeier (1979)]. The simple oxygen dissociation experiment itself can be described within the heterogeneous Adair model by making, for instance, both k_α's = 18 s^{-1} and both k_β's = 3.6 s^{-1}. In terms of an allosteric model, the experiments show that there is no significant T form for the half-liganded species during the time for oxygen dissociation, because equilibrium favors the R form and/or the R $\rightarrow$ T transition is slow, $t_{1/2}$ being tens of milliseconds. The detection of a rapid form by oxygen pulse, on the other hand, suggests a significant fraction of T form at equilibrium and/or a slow rate constant for the T $\rightarrow$ R transition. Thus, if the equilibrium constant for R $\rightleftharpoons$ T for half-ligation is not of the order of 1 (i.e., 0.2–5), then for the half-oxygenated species at least one of the conformational change rate constants must be quite slow.

The unusual oxygen association kinetics following photodissociation of the CO from HbCO are consistent with the existence of two different conformations for HbCO and HbO_2. A number of mechanisms were investigated in an attempt to account for the lower than first power dependence on O_2 concentration for the curves at all six oxygen concentrations. Three of these will be discussed briefly. (For the following models, we neglect heme heterogeneity only for simplicity of representation.) (1) Hb′ $\rightarrow$ Hb $\rightarrow$ HbO_2. In applying this model to data for all six oxygen concentrations, a marked lag phase is predicted, but none was observed. (2) Hb + O_2 $\rightleftharpoons$ HbO_2. As is well known, this mechanism can account for the lower than first power dependence on O_2 concentrations only for a reaction which does not go to completion. The minimum oxygen concentration employed, however, was 10 times the oxygen concentration for half-saturation. (3) Hb′ + O_2 $\rightleftharpoons$ Hb′O_2 $\rightarrow$ HbO_2. This model does not predict a lower than first power dependence on oxygen concentration. If our branching mechanism is correct, it suggests that the liganded CO conformation reverts with a half-time of about 1 ms to another conformation for which the O_2 association rate constant is about 30–40 times that for the CO conformation. At low oxygen concentrations, there is thus time for the Hb′ $\rightarrow$ Hb transition, followed by the more rapid binding reaction; at high oxygen concentrations, the binding of O_2 to the Hb′ form with the lower rate constant competes favorably with the Hb′ $\rightarrow$ Hb conformational transition. Accordingly, the apparent dependence of the oxygen binding rate would be less than first power in oxygen. In terms of our difference spectra this conformational change, Hb′ $\rightarrow$ Hb, appears to involve the movement of one tryptophan per heme from the solvent to a nonpolar environment.

The oxygen dissociation kinetics in the presence of CO were unusual for a cooperative hemoglobin in that the binding of CO could not compete with the dissociation of oxygen so as to maintain the protein in a fully liganded form. Observations at the HbO_2–HbCO isosbestic point showed nearly half of the hemes was unliganded during the reaction. This means that the maximum rate constant for CO binding is about 2×10^4 M^{-1} s^{-1}. The R form of the half-liganded species persists for tens of milliseconds during the ordinary dissociation reaction. Although this may be an R-form characteristic of HbO_2 and not be quite optimal for CO binding, the evidence lends support to the arguments to be presented in the following paper that the *Thyonella* hemoglobin lacks the Hb* (quickly reacting) form for CO binding.

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Kinetics of Carbon Monoxide Binding to the Cooperative Dimeric Hemoglobin from *Thyonella gemmata*. Analysis of Carbon Monoxide Equilibrium Results†

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ABSTRACT: The CO association reaction for the dimeric hemoglobin of *Thyonella gemmata* is biphasic with rate constants of 2×10^3 and 1×10^4 M^{-1} s^{-1}, among the slowest known for a hemoglobin. The kinetic heterogeneity almost certainly derives from differences in the α- and β-chain heme environments since 90% of the hemoglobin migrated as a single electrophoretic component and the more rapid kinetic phase contributed about 48% to the absorbance change. The activation enthalpies for the CO association reaction are 7.5 kcal/mol (rapid heme) and 9.0 kcal/mol (slow heme). The value for horse hemoglobin is 8.3–8.6 kcal/mol corresponding to a CO association constant of 1.7×10^5 M^{-1} s^{-1}. These findings suggest that the CO binding site in *Thyonella* hemoglobin is even more restricted than that in horse hemoglobin. Stopped-flow and flash-photolysis results were identical. Although the hemoglobin is cooperative in CO binding, there is no evidence for a quickly reacting (Hb*) form of the protein. Tandem flash-photolysis experiments show that the heme that is rapidly reacting toward CO is also the more rapid in oxygen association and dissociation. Stopped-flow studies on partially CO-saturated hemoglobin show that intermediate liganded forms are present in significant amounts and allow all equilibrium constants to be determined in a heterogeneous Adair model. The Adair model can account satisfactorily for all existing CO equilibrium and kinetic results and predicts that the dissociation of CO from half-liganded forms should be about 10 times faster than that from Hb-$(CO)_2$. A heterogeneous allosteric model using observed rate constants assumed to pertain to the R form gave $L = 150$ and $c = 0.0207$ and predicted a 48-fold enhancement in the CO off rate (T form vs. R form). If there exists a quickly reacting CO form (Hb*) with an association constant only 10 times that which is observed, but which reverts to the T form more rapidly than CO binds to Hb* (thus accounting for its nonappearance), $L = 15479$, $c = 0.00242$, the enhancement in the CO dissociation rate is 41, and the equilibrium ratio of R to T for half-liganded hemoglobin is 0.02. A larger value for the hypothetical Hb* association constant leads to an even larger value for the allosteric constant L.

The study of the kinetics of CO binding to a cooperative hemoglobin usually provides the clearest kinetic evidence for cooperativity. For tetrameric hemoglobins, evidence for a quickly reacting form (Hb* or R form) can be obtained by flash photolysis (Gibson, 1959a). By the same method, the rate of the Hb* to Hb transition can be measured (Gibson, 1959b; Sawicki & Gibson, 1977) and tetramer–dimer equilibrium constants can be obtained (Edelstein et al., 1970; Steinmeier & Parkhurst, 1975). For cooperative dimeric heme proteins, there may be no detectable Hb* form (Yang, 1974; Geraci et al., 1977). In the preceding paper (Steinmeier & Parkhurst, 1979), a study of the deoxygenation of HbO_2 in the presence of saturated CO (observed at the HbO_2–HbCO isosbestic wavelength) showed that the oxy-R form of *Thyonella* hemoglobin was not a quickly reacting form for the ligand CO or that the R → T process depopulated the R form of half-oxygenated hemoglobin before it could bind rapidly with CO. The present paper reports CO kinetic studies by stopped-flow and flash-photolysis experiments, which, in addition to the more conventional experiments, entailed the study of CO binding to intermediates generated by flash photolysis (tandem flash experiments) and to intermediates prepared by equilibration with CO. Carbon monoxide dissociation was studied by oxygen replacement.

Experimental Procedures

Procedures not dealt with in the preceding paper will be described below.

Carbon monoxide binding by stopped-flow experiments employed 10 μM hemoglobin, heme basis, before mixing, and CO concentrations which ranged, before mixing, from 90 to 940 μM. The reaction was studied in 50 mM potassium

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