

Trematode Hemoglobins Show Exceptionally High Oxygen Affinity

Laurent Kiger,* Aftab K. Rashid,* Nathalie Griffon,* Masoodul Haque,# Luc Moens,§ Quentin H. Gibson,[¶] Claude Poyart,* and Michael C. Marden*

*INSERM U473, 94276 Le Kremlin Bicêtre Cedex, France; #Department of Zoology, Patna University, Patna Bihar 800006, India;

§Department of Biochemistry, University of Antwerp, B 2610 Wilrijk, Belgium; and [¶]Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77005 USA

ABSTRACT Ligand binding studies were made with hemoglobin (Hb) isolated from trematode species *Gastrothylax crumenifer* (Gc), *Paramphistomum epiclitum* (Pe), *Explanatum explanatum* (Ee), parasitic worms of water buffalo *Bubalus bubalis*, and *Isoparorchis hypselobagri* (Ih) parasitic in the catfish *Wallago attu*. The kinetics of oxygen and carbon monoxide binding show very fast association rates. Whereas oxygen can be displaced on a millisecond time scale from human Hb at 25°C, the dissociation of oxygen from trematode Hb may require a few seconds to over 20 s (for Hb Pe). Carbon monoxide dissociation is faster, however, than for other monomeric hemoglobins or myoglobins. Trematode hemoglobins also show a reduced rate of autoxidation; the oxy form is not readily oxidized by potassium ferricyanide, indicating that only the deoxy form reacts rapidly with this oxidizing agent. Unlike most vertebrate Hbs, the trematodes have a tyrosine residue at position E7 instead of the usual distal histidine. As for Hb *Ascaris*, which also displays a high oxygen affinity, the trematodes have a tyrosine in position B10; two H-bonds to the oxygen molecule are thought to be responsible for the very high oxygen affinity. The trematode hemoglobins display a combination of high association rates and very low dissociation rates, resulting in some of the highest oxygen affinities ever observed.

INTRODUCTION

Trematodes are the parasitic worms inhabiting various body organs of vertebrates where oxygen might be scarce or intermittent, as in the bile ducts or stomach of ruminants or the swim bladder of fishes. Trematodes possess a cytoplasmic oxygen-binding hemoprotein of ~17 kDa (Haque et al., 1992; Rashid et al., 1993, 1997) distributed throughout the body. The absorption spectrum shows the presence of heme (Fe-protoporphyrin IX) as a prosthetic group in the globin (Haider and Siddiqi, 1976; Rashid et al., 1997).

The reports of anaerobic metabolism in trematodes raise questions about the physiological role of the trematode hemoglobins. Unlike human Hb, the trematode oxygen-binding hemoproteins are monomeric and are not involved in the transport of oxygen within a circulatory system. High oxygen affinity molecules would not release the oxygen rapidly, which might indicate a function, like that of the monomeric leghemoglobin found in the nodules on legumes, which maintains a low level of free oxygen. The high concentration of the trematode Hb suggests a role as a reservoir, such as the myoglobin in seals or whales. The trematodes spend part of their life cycle exposed to air before encountering snails as their first host; those arriving at their final destination in the buffalo stomach may still be exposed occasionally to higher oxygen levels. Because the term “myoglobin” implies a relation to muscle tissue, these

oxygen-binding hemoproteins of trematodes will be called hemoglobins.

Structural modifications in the heme pocket architecture modulate the ligand-binding affinity of hemoglobin molecules. Modifications in the hemoglobin function occur according to the physiological needs of animals (Poyart et al., 1992). The iron-oxygen complex in Hb *Ascaris* is stabilized by an additional hydrogen bond contributed by B10 Tyr (De Baere et al., 1994; Yang et al., 1995; Huang et al., 1996; Peterson et al., 1997). This leads to an increased oxygen affinity due to a lower rate of oxygen dissociation (Fig. 1). Leghemoglobins, on the other hand, have high oxygen affinity through high oxygen association rates, because of the large size of the heme pocket (Gibson et al., 1989).

The amino acid sequence analysis of hemoglobins from trematode species *Paramphistomum* and *Isoparorchis* shows that a tyrosine is present at B10 and E7 positions (Rashid et al., 1997), instead of the more common B10 Leu and E7 His. The mutant sperm whale myoglobin (SW Mb) with B10 tyrosine has low oxygen affinity (Gibson et al., 1993); however, a phenylalanine in position B10 increases the oxygen affinity and slows the rate of autoxidation of myoglobin (Carver et al., 1992). Substitution of the distal histidine by tyrosine in SW Mb makes the iron susceptible to oxidation (Springer et al., 1989).

Another barrier for oxygen and carbon monoxide binding to native myoglobin is the displacement of a water molecule from the distal pocket of deoxymyoglobin by disrupting the polar interactions with the distal histidine. Absence of a water molecule from the interior of domain I of Hb *Ascaris* may account for favorable energetics of oxygen binding (Yang et al., 1995).

Phylogenetic analysis based on the primary structure of trematode hemoglobin indicates that these are the most

Received for publication 17 December 1997 and in final form 27 April 1998.

Address reprint requests to Dr. Michael C. Marden, INSERM U473, 84 rue du General Leclerc, 94276 Le Kremlin Bicêtre Cedex, France. Tel.: 33-1-46-70-89-89; Fax: 33-1-46-70-64-46; E-mail: marden@kb.inserm.fr.

© 1998 by the Biophysical Society

0006-3495/98/08/990/09 \$2.00

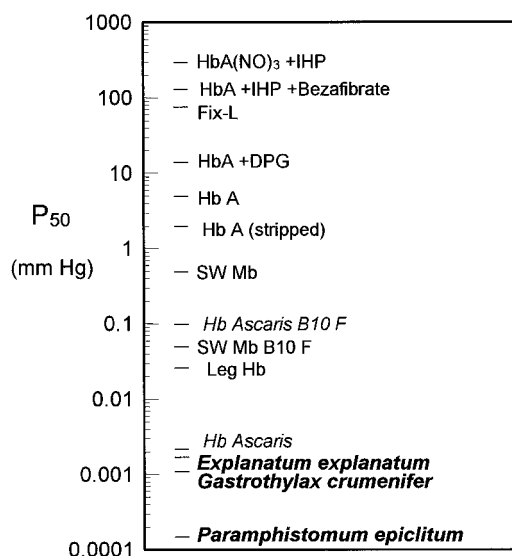


FIGURE 1 Oxygen affinity (partial pressure of oxygen, in mm Hg, for half-saturation) of various hemoproteins, at 25°C, pH 7. Hb *Ascaris* and the trematodes show the highest oxygen affinities (lowest P50 values).

primitive animal globins known today (Rashid et al., 1997). We report here the functional properties of hemoglobin from trematode species *Gastrothylax crumenifer* (*Gc*) and *Paramphistomum epiclitum* (*Pe*), parasitic in rumen, and *Explanatum explanatum* (*Ee*), parasitic in the bile ducts, of the water buffalo *Bubalus bubalis*, and *Isoparorchis hypselobagri* (*Ih*), parasitic in the swim bladder of the catfish *Wallago attu*.

MATERIALS AND METHODS

The water buffalo parasites (trematode species *Gc*, *Pe*, and *Ee*) were collected from a local slaughterhouse, and the catfish parasite *Ih* was from a local fish market, in Aligarh, India. The trematodes were immediately brought to the laboratory, washed extensively, and incubated in normal saline at 37°C for an hour to let the trematodes shed their eggs and gut contents. Trematodes were stored frozen at -70°C until sufficient material was collected.

The hemoglobin of trematodes was isolated and purified from whole worm homogenate in 50 mM Tris-HCl at pH 7.0, 1 mM phenylmethylsulfonyl fluoride (PMSF). The proteins in the homogenate were precipitated at 60% of ammonium sulfate saturation. The hemoglobin-containing fraction precipitating at 95% ammonium sulfate saturation was passed through a Sephadex G-75 column and eluted in the buffer used for homogenization of trematodes; this fraction, with a Soret band near 415 nm, was pooled and concentrated. Oxidation and ligand-binding kinetic studies were carried out on these samples.

Two main components were separated for trematode species *Gc*. After gel filtration chromatography, samples were subjected to ion exchange chromatography on CM52 cellulose. Isocratic elution by 10 mM phosphate buffer at pH 6 yielded four fractions. Isoelectric focusing showed two major bands and one minor band.

Oxidation studies

Autooxidation

The rate of autooxidation of oxyhemoglobins of trematodes was measured in 10 mM phosphate buffer (pH 7.0) at 37°C under air. The reaction was

started by adding trematode oxyhemoglobin to a final concentration of 10 μ M in the buffer solution, maintained at 37°C; spectra were recorded at regular intervals of time with an SLM-Aminco (DW2000) spectrophotometer. For the reference spectrum of the oxidized state, the hemoglobin was completely converted to its ferric form by equilibration under nitrogen, followed by the addition of potassium ferricyanide.

Potassium ferricyanide-mediated oxidation

To a solution of 10 μ M trematode hemoglobin under air, a 10-fold excess of potassium ferricyanide was added. The rate of oxidation reaction at room temperature was determined by analyzing the full spectra (HP 8453 spectrophotometer with diode array detection) versus time. For studies with deoxy-Hb, the sample was first flushed with nitrogen, and then a twofold excess of dithionite was added to remove the remaining oxygen, before the addition of a 10-fold excess of ferricyanide.

Oxygen binding kinetics

Oxygen association (k_{on})

Oxygen bimolecular on rates were measured at room temperature after photolysis with 10-ns laser pulses (Quantel, France). Hemoglobin concentration was 10 μ M (on heme basis) in 50 mM Tris-Cl buffer at pH 7.0.

Oxygen dissociation (k_{off})

Oxygen off rates were measured by stopped-flow spectrophotometry (Biologic, France) at 25°C. Hemoglobin concentration was 50 μ M on heme basis, in 50 mM Tris-Cl, pH 7.0 buffer. Molecular oxygen bound to trematode Hb was removed by mixing with 10 mM dithionite solution in 50 mM Tris-Cl buffer, pH 7.0. For the study at lower temperature, the full spectra were recorded versus time with a HP 8453 diode array spectrophotometer.

Carbon monoxide binding kinetics

Carbon monoxide association (l_{on})

Trematode hemoglobins (10 μ M on heme basis) were equilibrated with CO (1 atmosphere) and photodissociated with laser pulses of 10 ns. The recombination kinetics were followed at 436 nm, near the deoxy peak absorbance, in 50 mM Tris-Cl buffer at pH 7.0.

Carbon monoxide dissociation (l_{off})

The rate of carbon monoxide dissociation was studied by stopped flow. The carbon monoxide form of the trematode hemoglobins (50 μ M on heme basis) was reacted with air-equilibrated buffer (50 mM Tris-HCl at pH 7.0). The kinetics of the CO to oxy transition were followed at a wavelength of 415 nm.

The partition coefficient (M) for O₂ versus CO binding to the trematode hemoglobins was determined by direct competition, using a mixed atmosphere in a tonometer attached to an optical cuvette of 4 mm light path. The hemoglobin solution (1 ml at 10 μ M in heme) was first equilibrated under 1 atm CO. Known amounts of pure oxygen were then injected into the tonometer by a gas-tight (Hamilton) syringe. The hemoglobin solution was equilibrated with the gas phase by gentle shaking for 15 min at 25°C in a water bath. The absorption spectrum of the hemoglobin solution was recorded after each equilibration step and analyzed as a fraction of the initial Hb-CO spectrum and the final oxygenated form.

Geminate kinetics

The ps-ns geminate ligand recombination kinetics were measured after photodissociation by 7-ps pulses of 30 mJ (YLF laser, Continuum) as described by Shibayama et al. (1995). The pump probe technique was used with photolysis at 524 nm and detection (CCD camera from Spectra Source Instruments) with a continuum obtained by passing the probe pulse through a water cell.

RESULTS

The trematode organisms, flattened worms of length 5–10 mm, show a reddish color due to their Hb. The extractions and crude purifications yield red oxy-Hb solutions. The first observation is their resistance to oxidation; they remain in the oxy form on the laboratory bench for weeks and do not react rapidly with oxidants, such as ferricyanide. Although oxygen binds reversibly, it is difficult to remove without resorting to the addition of dithionite to eliminate the oxygen in solution.

Oxidation kinetics

The hemoglobin *Pe* has a rate of autoxidation of $\sim 0.004/\text{h}$ at 37°C , an order of magnitude slower than the autoxidation rate of sperm whale myoglobin (Brantley et al., 1993). The species *Gc* and *Ee* had rates of 0.017/h and 0.038/h, respectively. The *Ih* species, however, showed an anomalously rapid oxidation within a few hours.

Hbs with normal oxygen affinities, in their oxy or deoxy form, are oxidized rapidly by ferricyanide. The oxy-Hbs of *Gc*, *Ee*, and *Pe* show a resistance to oxidation by ferricyanide, whereas the deoxy forms were oxidized within seconds. The slow rate observed for the trematode oxy-Hbs

indicates that the induced oxidation must wait for oxygen dissociation.

Oxygen binding kinetics

The ligand binding characteristics of trematode Hbs are summarized in Table 1, along with the kinetic properties of other normal and mutant Hbs/Mbs cited in the literature. Fig. 2 shows the oxygen association kinetics of *Ee* and *Gc* Hb. Trematode hemoglobins show oxygen binding kinetics an order of magnitude faster than that of native sperm whale myoglobin. Oxygen dissociation kinetics of *Ee* and *Gc* Hbs (Fig. 3) show a 35-fold slower oxygen dissociation compared to SW myoglobin. For the case of species *Gc*, a second fraction showed an oxygen dissociation rate about six times faster than that of the major component.

The very slow oxygen dissociation suggests a higher activation energy. To determine this value, kinetics were measured for Hb *Pe* versus temperature (Fig. 4). Analysis via an Arrhenius plot (Fig. 4, *inset*) gave an energy for oxygen dissociation from *Pe* Hb of 27 kcal/mol. This is ~ 5 kcal/mol higher than typical values for Mb and Hb.

Carbon monoxide binding kinetics

Carbon monoxide association kinetics for binding to *Gc* and *Ee* Hbs are shown in Fig. 5. As compared to sperm whale myoglobin, an increase of ~ 100 -fold in the CO association rate is observed for trematode hemoglobins. CO dissociation kinetics (Fig. 6) show a 60-fold increase in rate for trematode hemoglobin relative to SW Mb.

TABLE 1 Kinetic constants of O_2 and CO reactions with some hemoglobins/myoglobins

Species	Residue		k_{on} ($\text{M}^{-1} \text{s}^{-1}$) $\times 10^{-6}$	k_{off} (s^{-1})	KO_2 (M) $\times 10^8$	l_{on} ($\text{M}^{-1} \text{s}^{-1}$) $\times 10^{-6}$	l_{off} (s^{-1})	KCO (M) $\times 10^8$	KO_2/KCO	References
	B10	E7								
<i>Physeter</i> (SW) Mb	Leu	His	15	14	93.	0.55	0.019	3.4	27	Egeberg et al. (1990)
Mutant SW Mb	Phe	His	21	1.4	6.7	0.22	0.006	2.7	2.4	Carver et al. (1992)
Mutant SW Mb	Leu	Tyr	—	—	—	0.41	0.062	15.	—	Springer et al. (1989)
Mutant SW Mb	Leu	Phe	74	10000	13500	4.4	0.054	1.2	11000	Springer et al. (1989)
Mutant SW Mb	Leu	Gly	140	1600	1142	5.8	0.038	0.66	1744	Rohlf et al. (1990)
<i>Ascaris</i> Hb	Tyr	Glu	2.8	0.013	0.36	0.3	—	—	—	Gibson and Smith (1965)
Mutant <i>Ascaris</i> Hb	Leu	Glu	9	5	45	0.75	—	—	—	De Baere et al. (1994)
Mutant <i>Ascaris</i> Hb	Phe	Glu	40	2	5	2.7	—	—	0.090	De Baere et al. (1994)
Trematodes										
<i>Paramphistomum epi.</i>	Tyr	Tyr	108	0.033	0.03	28.	—	—	—	Rashid et al. (1997)
<i>Gastrothylax crum.</i>	Tyr	Tyr	205	0.4	0.19	73.	1.2	1.6	0.119	This study
<i>Explanatum exp.</i>	?	?	120	0.4	0.33	33.	1.2	3.6	0.092	This study
<i>Dicrocoelium</i> Hb	Tyr	Tyr	300	30	10	110.	0.65	0.059	17	Di Iorio et al. (1985)
Leg-Hb (soybean)	Tyr	His	116	5.5	4.8	13.	0.01	0.0078	61	Appleby et al. (1983)
<i>Aplysia</i> Mb	Leu	Val	15	70	467	0.5	0.02	4.00	117	Wittenberg et al. (1965)
<i>Glycera</i> Hb II	Leu	Leu	186	1800	968	27.	0.04	0.14	6480	Rohlf et al. (1990)
<i>Chironomus</i> Hb	Leu	His	300	218	73	0.27	0.095	35.	2	Amiconi et al. (1972)

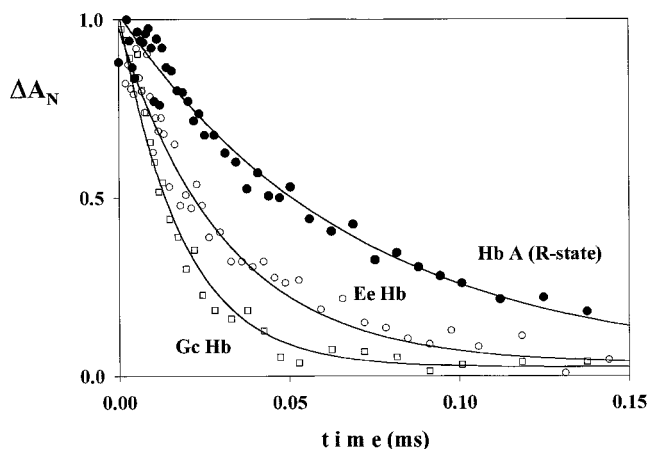


FIGURE 2 Bimolecular oxygen recombination kinetics after photodissociation of trematode Hbs *Gastrothylax crumenifer* (Gc) and *Explanatum explanatum* (Ee) and human Hb A. Experimental conditions were 25°C, 50 mM phosphate buffer at pH 7.

Geminate kinetics

As observed for other species with rapid bimolecular kinetics, the geminate ligand recombination is also rapid relative to other Hbs (Fig. 7). The high rate results in a decreased yield for the fraction that escape and rebind via the bimolecular phase, with a yield of ~ 0.5 for CO at 25°C and less than 0.02 for oxygen.

Spectral shifts at short times after photodissociation of CO, O₂, or NO are shown in Fig. 8. At short times, the spectra show a transient bleaching of the Soret peak, most of which is recovered in the first few picoseconds. This is a general feature of many myoglobins.

With CO there is a significant recombination reaction that is about two-thirds complete in 2.5 ns (Fig. 8 B). This is faster than for human Hb and about as rapid as Hb Zurich, in which a strong cage effect apparently maintains the

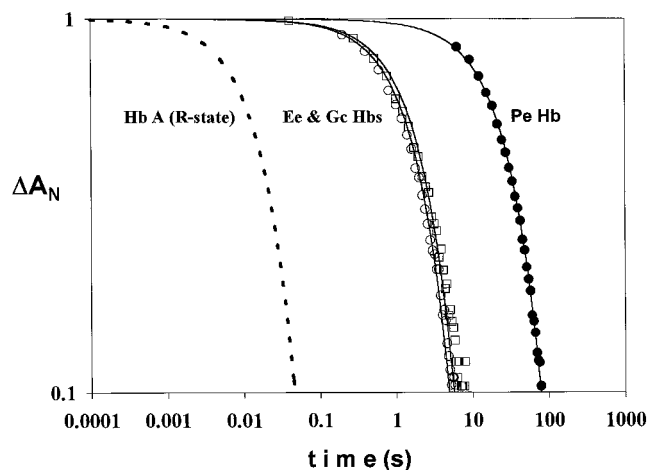


FIGURE 3 Oxygen dissociation kinetics of the trematode Hbs indicated, and for human Hb A. Because of the wide variation in rates, a logarithmic scale for the time (in seconds) is used. Experimental conditions were 25°C, pH 7.

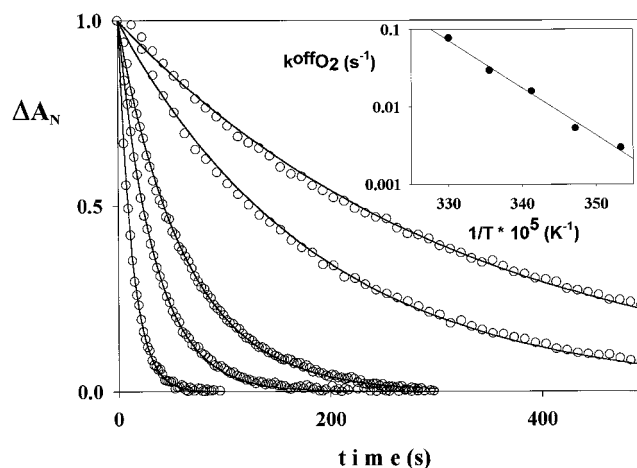


FIGURE 4 Oxygen dissociation kinetics of *Paramphistomum epiclitum* (Pe) Hb at pH 7, at the temperatures indicated. Analysis of the rate versus temperature yields an activation energy of 27 kcal/mol (inset).

ligand close to the iron atom. The kinetic traces for oxygen also show an unusually rapid recombination reaction. NO recombined completely by 100 ps, so the longer time trace (Fig. 8 F) is relatively flat.

DISCUSSION

The principal distinguishing characteristic of the trematode hemoglobins is a very low oxygen dissociation rate. These primitive species combine a high association rate with oxygen dissociation requiring seconds to obtain the very high affinities (Fig. 4). The trematodes may survive under anaerobic conditions, yet the Hb may be their most abundant protein. This raises the question about the physiological role of the trematode Hb. A high-affinity, noncirculating Hb would not provide an efficient oxygen delivery system. Two functional roles may be considered. The high oxygen affin-

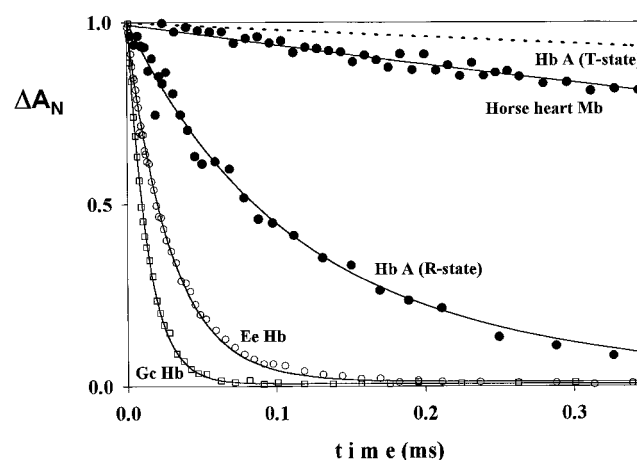


FIGURE 5 Bimolecular CO recombination kinetics, after photodissociation at 25°C, pH 7. The trematode Hb (Gc and Ee) kinetics are much faster than those for horse Mb or the high-affinity conformation (R-state) of human Hb.

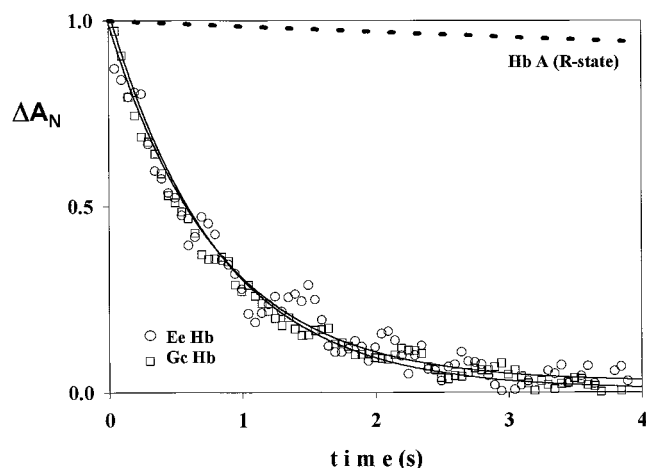


FIGURE 6 CO dissociation kinetics of trematode Hbs *Gc* and *Ee* at 25°C, pH 7, compared to that of human Hb.

ity suggests an oxygen-capturing role like that of Lb in nitrogen-fixing plant nodules. Within the host, the occasional exposure of the trematodes to air would suggest an alternative role for oxygen storage, like that of Mb; the low off rate would then regulate a slow release of oxygen during periods of low oxygen availability.

Oxidation kinetics

The hemoglobins of trematode species in this study, except that of *Isoparorchis hypselobagri* (*Ih*), show a reduced autoxidation rate. This property is consistent with the report that high oxygen affinity globins have low autoxidation rates (Brantley et al., 1993). Studies of mutant myoglobins have shown a relation between the autoxidation rate and the size of the B10 residue, although this residue is not critical for maintaining the structural integrity of the heme pocket (Carver et al., 1992). Consistent within this correlation, the second fraction of species *Gc*, with a higher off rate, showed a higher oxidation rate.

The hemoglobin of trematode *Isoparorchis* (*Ih*) shows an increased tendency toward autoxidation. In human Hb or

Mb, the distal His inhibits autoxidation by stabilizing the bound oxygen and restricting the entry of a water molecule into the heme cavity (Carver et al., 1992). Mutant myoglobin with distal Arg shows an increased rate of autoxidation and high affinity for CO. This mutant has an open distal pocket, due to the ligation of Arg side chain with one of the heme propionates, permitting easy entry of the solvent. The main criterion may therefore be the stability of the heme pocket; if the system is open to the solvent or allows dissociation of the heme group, a higher oxidation rate is observed. Point mutations in the heme pocket tend to perturb the carefully designed structure; however, an alternative pocket structure, as observed for the trematodes, may also provide a stable oxygen-binding system. In both human and trematode Hb, the distal residue plays a critical role; despite the occurrence of different amino acids in that position (His in most mammals, Tyr in trematode Hb, or Gln for elephant Mb), one cannot change only that residue and expect to maintain a fully functional heme pocket. Mutant SW myoglobin with Tyr at E7 position is reported to be highly unstable because of an increased tendency toward autoxidation (Springer et al., 1989). Thus single mutations may greatly perturb the heme pocket, and a larger remodeling of the pocket is required to accommodate a different distal residue.

Optical spectroscopy studies of *Dicrocoelium dendriticum* (*Dd*) Hb have shown that reduction of cyano-met Hb complex leads to the formation of the final deoxy-Hb spectrum, through an intermediate state, indicating the slow expulsion of the distal (E7) Tyr from the heme pocket (Lecomte et al., 1989).

The oxidation reaction of *Pe* oxy-Hb by ferricyanide is very slow, in contrast to the trematode deoxyhemoglobins. This indicates that the ligand bound form is protected from attack by the oxidizing agent. The protection is more obvious for the trematode species relative to human Hb, because of the very low dissociation rate of oxygen. It appears that in general, only the deoxyhemoglobins are oxidized rapidly by ferricyanide.

Kinetics of reaction with oxygen

The oxygen kinetic and equilibrium parameters of trematode hemoglobins are shown in Table 1. Trematode hemoglobins show high on rates and very low off rates, resulting in an extremely high oxygen affinity. The k_{on} rate reported for *Dicrocoelium* Hb (Di Iorio et al., 1985) is similar to the on rates of *Gc* and *Ee* Hbs. The oxygen dissociation from *Dd* Hb (Di Iorio et al., 1985) is normal, however, resulting in an oxygen affinity much lower than that of *Gc* and *Ee* Hbs.

There is a weak temperature dependence of the oxygen on rates; however, the oxygen off rates are quite sensitive to temperature, with an energy of activation of 28 kcal/mol. The trematode hemoglobins do not show cooperativity and remain monomeric. The Hb of *Dd* also shows a Hill coef-

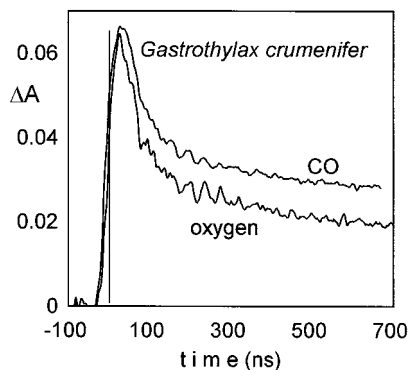


FIGURE 7 Geminate recombination kinetics of CO and oxygen to *Gastrothylax crumenifer* (*Gc*) Hb.

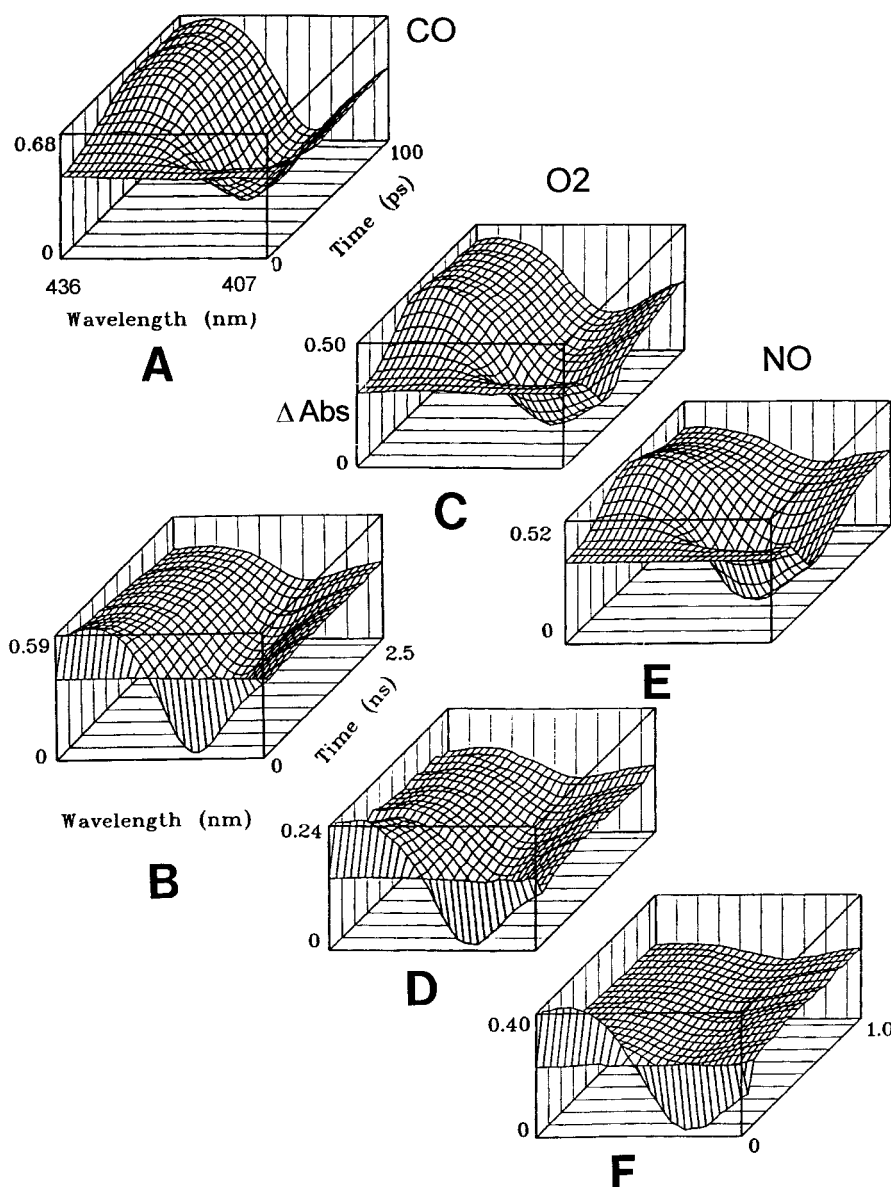


FIGURE 8 Spectral shifts during geminate reactions of CO (A and B), oxygen (C and D), and NO (E and F) to the trematode Hb of *Paramphistomum epiclitum* (Pe).

ficient value of 1 (Sick and Gersonde, 1985). Other high oxygen affinity proteins have low P_{50} values due to a low oxygen dissociation rate, like that of Hb *Ascaris* (Davenport, 1949), or to a high oxygen association rate, like that of leghemoglobin (Wittenberg et al., 1986). The trematode hemoglobins achieve an extremely high oxygen affinity by combining the two mechanisms of rapid association and slow dissociation of oxygen.

The fast bimolecular ligand binding rates of trematode hemoglobins are close to the value for the diffusion limit (Marden et al., 1986). There is thus apparently little resistance by the protein matrix to ligand movement. The association rates for myoglobin containing Gly at E7 position, chelated protoheme in soap micelles, and leghemoglobins are also very high, because of an "open" distal pocket (Mims et al., 1983).

Computer modeling studies on the *Pe* Hb heme pocket show that tyrosine at the E7 position is capable of moving

toward the solvent or into the heme pocket; both positions are capable of hydrogen bond formation (Rashid et al., 1997). A ^1H NMR study on the *Pe* Hb heme pocket could not confirm if E7 Tyr is oriented into the pocket or toward the solution; however, the *Pe* Hb heme pocket appears to be compact (Zhang et al., 1997). The fast ligand association rates of trematode hemoglobins coupled with slow oxygen dissociation therefore depend on the hydrogen bonding to residues in the heme pocket. The three-dimensional structure of myoglobin reveals no pathways or channels for the entry and exit of oxygen molecule. Transient motions of the protein are required to allow access to the iron atom.

The lack of potential hydrogen bond donors in E7 Gly, Val, and Phe myoglobin mutants produces a large increase in oxygen dissociation rate (Springer et al., 1989). The Fe-O₂ complex might be stabilized by two hydrogen bonds originating from B10 and E7 Tyr (Rashid et al., 1997). This type of heme pocket structure would therefore account for

slow dissociation rates due to an extra stabilization of the oxygen molecule in the heme pocket. In normal myoglobin, protonation and outward movement of the His E7 side chain at low pH (Tian et al., 1993; Yang and Phillips, 1996) increase the rate of ligand binding.

Several studies on Hb *Ascaris* indicate the origin of an extra hydrogen bond from tyrosine B10, stabilizing the $\text{Fe}^{2+}\text{-O}_2$ complex (De Baere et al., 1994; Yang et al., 1995; Huang et al., 1996; Peterson et al., 1997). This results in a slow dissociation rate of oxygen, giving a high oxygen affinity to Hb *Ascaris*. Substitution of B10 leucine by tyrosine in sperm whale myoglobin decreases the oxygen affinity (Carver et al., 1992). This is thought to be due to the steric hindrance by the hydroxyl group of tyrosine.

The temperature dependence of the dissociation rate also indicates an additional binding energy. An energy of 28 kcal/mol was determined, as compared to 10–20 kcal/mol for human Hb or 20 kcal/mol for Mb (Antonini and Brunori, 1971). The additional energy probably corresponds to the additional H-bond with the B10 tyrosine residue. The *Pe* species and Hb *Ascaris* show an even lower oxygen dissociation rate, but with apparently the same number of bonds with the oxygen molecule. This is apparently due to small structural changes that allow a better distance or orientation for the H-bonds.

Hb *Lucina pectinata* also shows decreased oxygen dissociation rates for types II and III, which have a tyrosine in position B10, relative to type I with B10 phenylalanine (Kraus and Wittenberg, 1990; Kraus et al., 1990); however, the association rates are also decreased, leading to a similar affinity for all three types.

Thus as a general rule the B10 tyrosine decreases the oxygen off rate. An exception to the rule is the mutant of SW Mb with B10 phenylalanine, which has a higher oxygen affinity than for the mutation with B10 tyrosine. Thus a tyrosine or phenylalanine at position B10 may stabilize the Fe-O_2 bond, but only for specific distances and orientations. Similarly, different species of leg-Hb have either a phenylalanine or tyrosine in position B10, but without a decrease in the oxygen off rate as large as that of Hb *Ascaris* (Appleby et al., 1983; Gibson et al., 1989).

The presence of a water molecule in the heme pocket does not seem to be of critical importance. Certain myoglobin mutants that lack the water molecule linked to their heme (replacement of E7 histidine with leucine, valine, or phenylalanine) have off rates for oxygen accelerated by 300-, 460-, and 700-fold, but the on rates are accelerated by only a factor of 6, 7, and 5, respectively (Quillin et al., 1993; Springer et al., 1989). This indicates that these hemoproteins may have no H-bonds with the oxygen ligand. On the other hand, *Aplysia limacina* Mb, which lacks a heme-linked water molecule, displays a high oxygen affinity due to a high on rate and a slow off rate (Conti et al., 1993; Wittenberg et al., 1965, 1972). The former is due to ready access to its open heme pocket, and the latter to the ability of arginine E10 to swing into the heme pocket and donate hydrogen bonds to heme ligands.

The role of hydrogen bonds in stabilizing the oxygen molecule has been demonstrated by synthetic model porphyrin compounds. Basket handle porphyrin compounds with an amide group attached to the aliphatic carbon chain show a higher affinity than those with an ether group. The increase in oxygen affinity is due mainly to the decrease in dissociation rates. Compounds derived from picket fence pocket porphyrin compounds by substituting an *m*-hydroxyphenyl substituent for one of the *t*-butyl groups in a picket show a substantial decrease in oxygen dissociation rates (Lavalette et al., 1990). Formation of a hydrogen bond between oxygen and the proton of the phenol-substituted picket might be responsible for this effect (Momenteau, 1996). The dipolar nature of the Fe-oxygen complex is important for the formation of the hydrogen bonds, and will also be influenced by the basicity of the fifth (proximal) iron ligand (Chang and Traylor, 1975).

Kinetics of reaction with CO

The Hbs of trematodes with distal tyrosine show fast association and dissociation kinetics for CO, but the overall affinity is close to that of normal myoglobins. The easy migration of CO through the heme pocket indicates the presence of a large or flexible heme cavity. The affinity for CO is governed mainly by the steric hindrance and the polar nature of the distal heme residue. The kinetics of CO binding to Dd Hb (Di Iorio et al., 1985) are similar to those for the trematodes.

Myoglobins substituted at the E7 position may show an increase in the CO on rate by over an order of magnitude, whereas the off rate is little affected (Rohlf et al., 1990). Substitution of Tyr at the E7 position, however, results in a fourfold decrease in the on rate, presumably because of increased hindrance; this is the only myoglobin mutant known to have decreased CO affinity (Springer et al., 1989). The phenyl side chain of the Tyr E7 mutant myoglobin is also known to be liganded to the heme group in the oxidized state, thus decreasing the volume of the heme cavity. Studies on synthetic heme compounds do not show a dipolar distal effect on the carbon monoxide binding (Chang and Traylor, 1975; Traylor and Berzini, 1980).

Partition coefficient

The affinity of heme proteins for carbon monoxide relative to oxygen is expressed in terms of the partition coefficient (*M*). Trematode hemoglobins show an extremely low coefficient value as compared to normal vertebrate myoglobins. The *M* values below 1 of trematode hemoglobins are due to an increased oxygen affinity rather than a decrease in carbon monoxide affinity. The carbon monoxide affinity of trematode hemoglobins is similar to that of normal vertebrate myoglobins. Fast entry of ligands into the trematode hemoglobin distal pocket is compensated for by a rapid dissociation for CO, but not for oxygen. In this case, an

extra stabilization of Fe-O₂ complex has been proposed. Hb *Ascaris*, which is also known for its extreme stability of the oxy state, shows a low partition coefficient similar to that of trematode hemoglobins (De Baere et al., 1994).

The structure of the distal pocket in heme proteins favors the binding of oxygen over carbon monoxide by favoring a bent geometry of the Fe-O₂ bond and forming a hydrogen bond with oxygen molecule with the distal histidine. In trematode hemoglobins, the stabilization of oxygen molecule, but not the apolar CO, is further enhanced by an additional hydrogen bond contributed by the B10 tyrosine, resulting in a further decrease in the *M* value.

At the other extreme, the partition coefficient for certain model heme compounds is more than 100 times that of human hemoglobin (Mometeau, 1996). Certain hemoproteins may lack the usual hydrogen bond, such as *Glycera* Hb (E7 Leu) with an increased CO and decreased O₂ affinity (Parkhurst et al., 1980), and *Aplysia* Mb (E7Val) with normal CO affinity but decreased O₂ affinity (Wittenberg et al., 1965); they exhibit 27-fold and 4-fold higher *M* values, respectively, as compared to native sperm whale myoglobin. In both cases, lack of a potential H-bond donor increases the O₂ dissociation rates, thereby increasing the *M* value.

CONCLUSIONS

The trematode hemoglobins are characterized by very high oxygen affinities, due mainly to low oxygen dissociation rates. As for Hb *Ascaris*, this enhanced affinity is due to an additional hydrogen bond with the bound oxygen via residue tyrosine B10, and therefore leads to a lower oxygen dissociation rate and higher activation energy for the dissociation rate. As for Lb, but not Hb *Ascaris*, the trematodes display high oxygen association rates; the combination of these two effects leads to some of the highest oxygen affinities ever observed.

This research was supported in part by INSERM; the University of Antwerp, Belgium; the Fund for Joint Basic Research, Flanders (grant No. 2.0023.94); and the U.S. National Institutes of Health (GM 14276). AKR was supported by a postdoctoral fellowship from INSERM.

REFERENCES

- Amiconi, G., E. Antonini, M. Brunori, H. Formanek, and R. Huber. 1972. Functional properties of native and reconstituted hemoglobins from *Chironomus thummi thummi*. *Eur. J. Biochem.* 31:52–58.
- Antonini, E., and M. Brunori. 1971. Hemoglobin and Myoglobin in Their Reactions with Ligands. North-Holland, Amsterdam.
- Appleby, C. A., J. H. Bradbury, R. J. Morris, B. A. Wittenberg, J. B. Wittenberg, and P. E. Wright. 1983. Leghemoglobin: kinetic, nuclear magnetic resonance and optical studies of pH dependence of oxygen and carbon monoxide binding. *J. Biol. Chem.* 258:2254–2259.
- Brantley, R. E., Jr., S. J. Smerdon, A. J. Wilkinson, E. W. Singleton, and J. S. Olson. 1993. The mechanism of autooxidation of myoglobin. *J. Biol. Chem.* 268:6995–7010.
- Carver, T. E., R. E. Brantley, Jr., E. W. Sargleton, R. M. Ardini, M. L. Quillin, G. N. Phillips, Jr., and J. S. Olson. 1992. A novel site directed mutant of myoglobin with an unusually high O₂ affinity and low autooxidation rate. *J. Biol. Chem.* 267:14443–14450.
- Chang, C. K., and T. G. Traylor. 1975. Kinetics of oxygen and carbon monoxide binding to synthetic analogues of the myoglobin and hemoglobin active sites. *Proc. Natl. Acad. Sci. USA.* 72:1166–1170.
- Conti, E., C. Moser, M. Rizzi, A. Mattevi, C. Lionetti, A. Coda, P. Ascenzi, M. Brunori, and M. Bolognesi. 1993. X-ray crystal structure of ferric *Aplysia limacina* myoglobin in different liganded states. *J. Mol. Biol.* 233:498–503.
- Davenport, H. 1949. The hemoglobin of *Ascaris lumbricoides*. *Proc. R. Soc. Lond. Biol.* 136:265–270.
- De Baere, I., M. F. Perutz, L. Kiger, M. C. Marden, and C. Poyart. 1994. Formation of two hydrogen bonds from the globin to the heme linked oxygen molecule in *Ascaris* hemoglobin. *Proc. Natl. Acad. Sci. USA.* 91:1594–1597.
- Di Iorio, E. E., U. T. Meier, J. D. G. Smit, and K. H. Winterhalter. 1985. Kinetics of oxygen and carbon monoxide binding to liverfluke (*Dicrocoelium dendriticum*) hemoglobin. *J. Biol. Chem.* 260:2160–2164.
- Egeberg, K. D., B. A. Springer, S. G. Sligar, T. E. Carver, R. J. Rohlf, and J. S. Olson. 1990. The role of Val 68 (E11) in ligand binding to sperm whale myoglobin. *J. Biol. Chem.* 265:11788–11795.
- Gibson, Q. H., R. Regan, J. S. Olson, T. E. Carver, B. Dixon, B. Pohajdak, P. K. Sharma, and S. N. Vinogradov. 1993. Kinetics of ligand binding to *Pseudoterranova decipiens* and *Ascaris suum* hemoglobin and to Leu-29Tyr sperm whale myoglobin mutant. *J. Biol. Chem.* 268:16993–16998.
- Gibson, Q. H., and M. H. Smith. 1965. Rates of reaction of *Ascaris* haemoglobins with ligands. *Proc. R. Soc. Lond. Biol.* 163:206–214.
- Gibson, Q. H., J. B. Wittenberg, B. A. Wittenberg, D. Bogusz, and C. A. Appleby. 1989. The kinetics of ligand binding to plant hemoglobins. *J. Biol. Chem.* 264:100–107.
- Haider, S. A., and A. H. Siddiqi. 1976. Spectrophotometric analysis of hemoglobins of some digenetic trematodes and their hosts. *J. Helminthol.* 50:259–266.
- Haque, M., K. A. Rashid, M. S. Stern, P. K., Sharma, A. H. Siddiqi, S. N. Vinogradov, and D. A. Walz. 1992. Comparison of the hemoglobins of the platyhelminths *Gastrothylax crumenifer* and *Paramphistomum epiclitum* (Trematoda: Paramphistomatidae). *Comp. Biochem. Physiol.* 101B:673–676.
- Huang, S., J. Huang, A. P. Kloek, D. E. Goldberg, and J. M. Friedman. 1996. Hydrogen bonding of tyrosine B10 to heme bound oxygen in *Ascaris* hemoglobin. *J. Biol. Chem.* 271:958–962.
- Kraus, D. W., and J. B. Wittenberg. 1990. Hemoglobins of the *Lucina pectinata*/bacterial symbiosis. *J. Biol. Chem.* 265:16054–16059.
- Kraus, D. W., J. B. Wittenberg, and J. Peisach. 1990. Hemoglobins of the *Lucina pectinata*/bacterial symbiosis. II. An electron paramagnetic resonance and optical spectra study of the ferric proteins. *J. Biol. Chem.* 265:16054–16059.
- Lavalette, D., C. Tétreau, M. Mometeau, J. Mispelter, J. M. Lhoste, G. E. Wuenschell, and C. A. Reed. 1990. Laser photolysis investigations of ligand binding with models of the active site of respiratory hemoproteins: kinetic and thermodynamic aspects. *Laser Chem.* 10:297–318.
- Lecomte, J. T. J., J. D. G. Smit, K. H. Winterhalter, and G. N. La Mar. 1989. Structural and electronic properties of the liver fluke heme cavity by nuclear magnetic resonance and optical spectroscopy. *J. Mol. Biol.* 209:235–247.
- Marden, M. C., E. S. Hazard, III, and Q. H. Gibson. 1986. Protoheme-carbon monoxide geminate kinetics. *Biochemistry.* 25:2786–2792.
- Mims, M. P., A. G. Porras, J. S. Olson, R. W. Noble, and J. A. Peterson. 1983. Ligand binding to heme proteins. An evaluation of distal effects. *J. Biol. Chem.* 258:14219–14232.
- Mometeau, M. 1996. Chapter 4. In *Supramolecular Control of Structure and Reactivity*. A. D. Hamilton, editor. John Wiley and Sons, New York. 155–223.
- Parkhurst, L. J., P. Sima, and D. J. Goss. 1980. Kinetics of oxygen and carbon monoxide binding to the hemoglobins of *Glycera dibranchiata*. *Biochemistry.* 19:2688–2692.
- Peterson, E. S., S. Huang, J. Wang, L. M. Miller, G. Vidugiris, A. P. Kloek, D. E. Goldberg, M. R. Chance, J. B. Wittenberg, and J. M. Friedman. 1997. A comparison of functional and structural consequences of the

- tyrosine B10 and glutamine E7 motifs in two invertebrate hemoglobins (*Ascaris suum* and *Lucina pectinata*). *Biochemistry*. 36:13110–13121.
- Poyart, C., H. Wajcman, and J. Kister. 1992. Molecular adaptations of hemoglobin function in mammals. *Respir. Physiol.* 90:3–17.
- Quillin, M. L., R. M. Arduini, J. S. Olson, and G. N. Phillips, Jr. 1993. High resolution crystal structures of distal histidine mutants of sperm whale myoglobin. *J. Mol. Biol.* 234:140–155.
- Rashid, K. A., M. Haque, A. H. Siddiqi, M. S. Stern, P. K. Sharma, S. N. Vinogradov, and D. A. Walz. 1993. Purification and properties of the hemoglobins of the platyhelminth *Isoparorchis hypselobagri* (Trematoda: Isoparorchidae) and its host *Wallagu attu* (catfish). *Comp. Biochem. Physiol.* 106B:993–998.
- Rashid, A. K., M. L. Van Hauwaert, M. Haque, A. H. Siddiqi, I. Lasters, M. De Mayer, N. Griffon, M. C. Marden, S. De Wilde, J. Clauwaert, S. N. Vinogradov, and L. Moens. 1997. Trematode myoglobins, functional molecules with a distal tyrosine. *J. Biol. Chem.* 272:2992–2999.
- Rohlfs, R. J., A. J. Mathews, T. E. Carver, J. S. Olson, B. A. Springer, K. D. Egeberg, and S. G. Sligar. 1990. The effects of amino acid substitution at position E7 (residue 64) on the kinetics of ligand binding to sperm whale myoglobin. *J. Biol. Chem.* 265:3168–3176.
- Shibayama, N., T. Yonetani, R. M. Regan, and Q. H. Gibson. 1995. Mechanism of ligand binding to Ni(II)-Fe(II) hybrid hemoglobins. *Biochemistry*. 34:14658–14667.
- Sick, H., and K. Gersonde. 1985. Continuous gas-depletion technique for measuring O₂-dissociation curves of high-affinity hemoglobins. *Anal. Biochem.* 146:277–280.
- Springer, B. A., K. D. Geberg, S. G. Sliger, R. J. Rohlfs, A. J. Mathews, and J. S. Olson. 1989. Discrimination between oxygen and carbon monoxide and inhibition of autooxidation by myoglobin. *J. Biol. Chem.* 264:3057–3060.
- Tian, W. D., J. T. Sage, and P. M. Champion. 1993. Investigations of ligand association and dissociation rates in the “open” and “closed” states of myoglobin. *J. Mol. Biol.* 233:155–166.
- Traylor, T. G., and A. P. Berzins. 1980. Binding of O₂ and CO to hemes and hemoproteins. *Proc. Natl. Acad. Sci. USA*. 77:3171–3175.
- Wittenberg, J. B., C. A. Appleby, and B. A. Wittenberg. 1972. The kinetics of the reaction of leghemoglobin with carbon monoxide. *J. Biol. Chem.* 247:527–531.
- Wittenberg, B. A., M. Brunori, E. Antonini, J. B. Wittenberg, and J. Wyman. 1965. Kinetics of the reactions of *Aplysia* myoglobin with oxygen and carbon monoxide. *Arch. Biochem. Biophys.* 111:576–579.
- Wittenberg, J. B., B. A. Wittenberg, Q. H. Gibson, J. T. Michael, and C. A. Appleby. 1986. The kinetics of the reactions of *Parasponia andersonii* hemoglobin with oxygen, carbon monoxide and nitric oxide. *J. Biol. Chem.* 261:13624–13631.
- Yang, F., and G. N. Phillips, Jr. 1996. Crystal structures of CO-, deoxy- and met myoglobins at various pH values. *J. Mol. Biol.* 256:762–774.
- Yang, J., A. P. Klock, D. E. Goldberg, and F. S. Mathews. 1995. The structure of *Ascaris* hemoglobin domain I at 2 Å resolution: molecular features of oxygen avidity. *Proc. Natl. Acad. Sci. USA*. 92:4224–4228.
- Zhang, W., K. A. Rashid, M. Haque, A. H. Siddiqi, S. N. Vinogradov, L. Moens, and G. N. La Mar. 1997. Solution of ¹H NMR structure of the heme cavity in the oxygen avid molecule from the trematode *Paramphistomum epiclitum*. *J. Biol. Chem.* 272:3000–3006.