Hemoglobins of the *Lucina pectinata/Bacteria Symbiosis*

I. MOLECULAR PROPERTIES, KINETICS AND EQUILIBRIA OF REACTIONS WITH LIGANDS*

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Three hemoglobins have been isolated from the symbiont-harboring gill of the bivalve mollusc *Lucina pectinata*. Oxyhemoglobin I (Hb I), which may be called sulfide-reactive hemoglobin, reacts with hydrogen sulfide to form ferric hemoglobin sulfide in a reaction that may proceed by nucleophilic displacement of bound superoxide anion by hydrosulfide anion. Hemoglobins II and III, called oxygen-reactive hemoglobins, remain oxygenated in the presence of hydrogen sulfide. Hemoglobin I is monomeric; Hb II and Hb III self-associate in a concentration-dependent manner and form a tetramer when mixed. Oxygen binding is not cooperative. Oxygen affinities are all nearly the same, \( P_{50} = 0.1 \) to \( 0.2 \) Torr, and are independent of pH. Combination of Hb I with oxygen is fast; \( k_{\text{on}} \) (estimated) \( 100-200 \times 10^8 \text{M}^{-1} \text{s}^{-1} \). Combination of Hb II and Hb III with oxygen is slow: \( k_{\text{on}} = 0.4 \) and \( 0.3 \times 10^9 \text{M}^{-1} \text{s}^{-1} \), respectively. Dissociation of oxygen from Hb I is fast relative to myoglobin: \( k_{\text{off}} = 61 \text{ s}^{-1} \). Dissociation from Hb II and Hb III is slow: \( k_{\text{off}} = 0.11 \) and \( 0.08 \text{ s}^{-1} \), respectively. These large differences in rates of reaction together with differences in the reactions of carbon monoxide suggest differences in configuration of the distal heme pocket. The fast reactions of Hb I are comparable to those of hemoglobins that lack distal histidine residues. Slow dissociation of oxygen from Hb II and Hb III suggest that a distal residue may interact strongly with the bound ligand. We infer that Hb I may facilitate delivery of hydrogen sulfide to the chemoautotrophic bacterial symbiont and Hb II and Hb III may facilitate delivery of oxygen.

The midpoint oxidation-reduction potential of the ferrous/ferric couple of Hb I, \( 103 \pm 8 \text{mV} \), was independent of pH. Potentials of Hb II and Hb III were pH-dependent. At neutral pH all three hemoglobins have similar midpoint potentials.

The rate constant for combination of ferric Hb I with hydrogen sulfide increases 3000-fold from pH 10.5 to 5.5, with apparent pK 7.0, suggesting that undissociated hydrogen sulfide is the attacking ligand. At the acid limit combination of ferric Hb I with hydrogen sulfide, \( k_{\text{on}} = 2.3 \times 10^9 \text{M}^{-1} \text{s}^{-1} \), is 40-fold faster than combination with ferric Hb II or myoglobin. Dissociation of hydrogen sulfide from ferric Hb I, \( k_{\text{off}} = 0.22 \times 10^{-3} \text{ s}^{-1} \), is 70-fold slower than dissociation from Hb II, \( k_{\text{off}} = 17 \times 10^{-3} \text{ s}^{-1} \), Hb III, and myoglobin. The affinity of ferric Hb I for sulfide, \( K' = 4 \text{mM} \) at pH 7.5, is 4000-fold greater than those of ferric Hb II, \( K' = 15 \mu \text{M} \) at pH 7.5, Hb III, and myoglobin. Ferric Hb I sulfide is reduced to ferrous hemoglobin by hydrogen sulfide or dithionite without prior dissociation of the ligand. In light of the very slow dissociation of bound sulfide we suggest that, if Hb I delivers sulfide to the bacterial symbiont, reduction of the heme iron may precede disengagement of the bound ligand.

Chemooautotrophic bacteria, which oxidize hydrogen sulfide, methane, or other reduced substances and fix carbon dioxide into hexose, form the food base of dense animal populations in environments where hydrogen sulfide or other reductants meet oxygen in disequilibrium (1). Among these environments are the oceanic hydrothermal vents, of which the Galápagos vents are the first discovered. Those members of the vent fauna, the giant tube worm (2), the giant clam (3) and a snail (4), which interiorize the bacteria as intracellular symbionts lack, or nearly lack, mouth and guts and depend almost entirely on the symbionts for their supply of organic carbon (1). The gigantic size these animals attain attests to the effectiveness of the symbioses. Less flamboyant, but perhaps representing as large a biomass, are worldwide populations of symbiont-harboring clams and vestimentiferan worms exploiting disequilibrium mixtures of oxygen and reductants in coastal and deep sediments (5).

Hemoglobin is a nearly constant feature of symbioses between invertebrates and chemoautotrophic bacteria (6). Circulating hemoglobin brings oxygen and hydrogen sulfide into intimate contact with the bacteria-harboring cells of the giant tube worm (7). Cytoplasmic hemoglobins, (historically called myoglobins) occur at high concentration in the bacteria-housing gills of many (6, 8) but not all (9) mollusces which support symbioses with bacteria.

Cytoplasmic hemoglobin is likewise a nearly invariant feature of symbioses between plants and intracellular, nitrogen-fixing rhizobia or actinomycetes (10, 11). Leghemoglobin (legume hemoglobin) is obligatorily required for effective nitrogen fixation by bacterial symbionts in the intact legume root nodule (10, 12, 13), and leghemoglobin-bound oxygen supports oxidative phosphorylation by isolated symbionts far more effectively than does dissolved oxygen (10, 12, 13). We perceive an analogy between the roles played by cytoplasmic hemoglobins in the molluscan and plant symbioses and suggest that molluscan gill hemoglobin may supply oxygen to the bacterial symbiont just as leghemoglobin supplies oxygen to the nitrogen-fixing symbiont of the root nodule.

The bacterial partner of the molluscan symbioses must be supplied with hydrogen sulfide as well as with oxygen. Spectroscopic observation of the living, bacteria-harboring gill of the clam *Solemya velum* exposed to hydrogen sulfide (at micromolar concentration) shows that about one-half of the...
hemoglobin is converted rapidly and reversibly to ferric hemoglobin sulfide in situ, the balance remaining oxygenated (14). In another species, Solemya reidi, the bacteria-harboring domain and the mitochondria-rich ciliated domain of the gill may be observed separately. The reaction with sulfide is confined to the bacteria-harboring domain.1 Hemoglobin in the ciliated domain of the gill and in other tissues of the clam remains solely oxygenated or deoxygenated. These observations suggest that oxygen-reactive hemoglobins are widely distributed and that the sulfide-reactive hemoglobin is found only where it may aid the sulfide supply to the symbiont.

The clam, Lucina pectinata, inhabiting sulfide-rich coastal sediments, was chosen to study the role of cytoplasmic hemoglobins in the symbioses between clams and intracellular chemosynthetic bacteria. The gill housing the bacteria is massive, and the cytoplasmic hemoglobin concentration is large, approaching 1.5 mM (as heme) (6, 15, 16). In this study, we describe the properties of the three cytoplasmic hemoglobins which may be isolated from the Lucina gill and try to discern those properties of each hemoglobin which favor reaction with either oxygen or hydrogen sulfide. We find that the two hemoglobins which resist reaction with hydrogen sulfide, “oxygen-reactive hemoglobins,” display unusual kinetics in their reactions with oxygen and carbon monoxide and unusual acid/alkaline transitions in the ferric state. We suggest that the distal ligand to the heme of these proteins may be a residue, other than histidine, which binds strongly to the bound oxygen molecule and also forms a stable bond to the heme in the alkaline ferric state.

We find that the hemoglobin which reacts with hydrogen sulfide (in the presence of oxygen) to form ferric hemoglobin sulfide, the “sulfide-reactive hemoglobin,” combines rapidly with oxygen and, when ferric, achieves extraordinary affinity for sulfide by rapid combination together with very slow dissociation. These properties are consistent with the possibility that the distal histidine residue commonly found in hemoglobins is lacking in the sulfide-reactive hemoglobin.

Preliminary accounts of parts of this work have appeared (17, 18).

MATERIALS AND METHODS

RESULTS

Recoveries of Isolated Proteins—The elution profile of the initial Sephadex G-75 column (Fig. 1) shows an asymmetric high molecular weight hemoglobin band (Hb II and III) and a symmetric band of lower molecular weight hemoglobin (Hb I). In addition, a cysteine-rich protein peak, not encountered in other hemoglobin-containing tissues, immediately precedes Hb I and is not completely resolved from it. A yellow, iron-containing fraction is eluted much later.

The low molecular weight oxyhemoglobin, Hb I, is well resolved on a column of DEAE-Sepharose from a small amount of ferric hemoglobin and from the cysteine-rich protein (Fig. 2A). Hemoglobin I is probably the same as Read’s hemoglobin I (16).

The two higher molecular weight oxyhemoglobins, Hb II and Hb III, are resolved on a column of DEAE-Sepharose developed with a gradient of NaCl (Fig. 2B). These are probably the same as Read’s Hemoglobins 2 and 3 (16).

Recoveries of the three hemoglobins, estimated as pyridine hemochromogen, were: Hb I, 0.58 mmol kg−1; Hb II, 0.50 mmol kg−1; Hb III, 0.50 mmol kg−1 wet weight tissue. Recovery of the cysteine-rich protein was 0.66 mmol kg−1 wet weight tissue estimated taking ε480 = 47 at 280 nm, based on amino acid composition expressed to the nearest integer. Recovery of iron in the yellow fraction, determined by atomic absorption spectroscopy, was 0.44 mmol kg−1 wet weight tissue.

Identity of the Heme—The heme of each protein was identified as iron protoporphyrin IX by the characteristic optical spectrum of the pyridine hemochromogens with absorbance maxima at 557 and 524 nm.

Purity of Isolated Proteins—Each hemoglobin and the cysteine-rich protein was homogeneous; only single bands were observed on SDS-polyacrylamide gel electrophoresis.

Subunit Molecular Size and Heme Content—The apparent subunit sizes of the four proteins, determined by SDS-polyacrylamide gel electrophoresis, by gel filtration chromatography and calculated from amino acid composition, are gathered in Table I. Molecular sizes calculated from amino acid content, considered the more accurate estimate, tended to be slightly larger than estimates based on gel filtration. The subunits of the two high molecular weight hemoglobins, Hb II and Hb III, differ in apparent size, and both in turn differ from Hb I. The molecular weight of Hb I, 14,484, in good agreement with Read’s previous determination (16), is notably small. The minimum molecular size of the cysteine-rich protein, deduced from amino acid analysis alone is in reasonable agreement with that determined by SDS-polyacrylamide gel electrophoresis or gel filtration chromatography (Table I); we accept a value of 252 residues, corresponding to a molecular weight of 26,294.

Amino Acid Composition—The amino acid composition of Hb I, II, and III and the cysteine-rich protein are collected in Table II. Hemoglobin I contains 3 histidine residues. Hemoglobin II and III each have 5 histidine residues. Hemoglobin I contains 3 cysteine residues that react as sulfhydryls. Hemoglobin II and III contain 2 and 3 cysteine residues, respectively, that do not react as sulfhydryls indicating that the sulfhydryl groups are buried within the protein and/or present as disulfide bonds. The cysteine-rich protein contains 11 cysteine residues as determined by amino acid analysis and 12 sulfhydryl groups as determined colorimetrically.

Table I

| Protein | Apparent subunit size (kDa) | SDS-PAGE | Gel filtration | Amino acid composition | Oligomer at high concentration |
|---------|-----------------------------|----------|----------------|------------------------|-------------------------------|
| Hb I    | 14,484                      | 13,400   | 13,000         | 14,443                 | Monomer                      |
| Hb II   | 15,700                      | 15,100   | 17,700         | 16,128                 | Tetramer                      |
| Hb III  | 15,100                      | 16,700   | 16,700         | 17,762                 | Dimer                        |
| Cysteine-rich protein | 26,200 | 19,000 | 12,000 | 26,294 | Monomer |

1 Portions of this paper (including "Materials and Methods," part of "Results," Figs. 1-5, 7-10, and Tables II and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: Hb, hemoglobin; Mb, myoglobin; Lb, legume hemoglobin; HbO2, oxyhemoglobin; HbCO, carbon monoxide hemoglobin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
number of sulfhydryl groups detected was the same after reduction with dithiothreitol indicating that disulfide bonds were not present.

Self-association of Subunits of Hb II and Hb III—Oxyhemoglobin I behaves as a monomer to gel filtration chromatography on Superose columns at all concentrations examined, 4 μM to 3 mM heme in the applied sample (Fig. 3). Oxyhemoglobins II and III behave as apparent monomers at low concentration and form aggregates at higher concentration. The apparent molecular size of Hb II increases with increasing hemooglobin concentration tending toward an oligomer of more than four subunits, at 4 mM applied concentration (Fig. 3). The apparent molecular size of oxyhemoglobin III does not obey a simple dissociation equilibrium. The major eluted peak tends toward 34,000 daltons, corresponding to dimer, at more than millimolar applied concentration (Fig. 3). Minor peaks, following the major peak at low concentration and proceeding it at high concentration, suggest formation of larger oligomers at high protein concentration (not illustrated). An equimolar mixture of oxyhemoglobin II and III eluted as a single symmetric peak at all concentrations. The apparent molecular size followed a sigmoidal curve as a function of concentration, tending toward a limit near 70,000 daltons, corresponding to tetramer, at 6 mM applied concentration (Fig. 3).

The cysteine-rich protein behaves as an apparent monomer at all concentrations of the applied sample, 5 to 100 μM.

Optical Spectra—Spectral constants of ferric Lusica hemoglobins and their derivatives are collected in Table III. Spectra of all three Hbs are very nearly the same. Spectra in the visible region of the carbon monoxide derivatives of the three Lusica hemoglobins, in common with other cytoplasmic clam hemoglobins and with leghemoglobins, exhibit only a single symmetric α maximum, centered near 568 nm (Fig. 4). Vertebrate myoglobins exhibit an additional partly resolved α maximum near 578 nm.

Oxygen Combination Rates—Oxygen combination with Hb I was too fast to measure in the stopped flow apparatus. Oxygen combination with Hb II and Hb III was slow and independent of pH from 6 to 9. The rate constants are collected in Table IV.

Oxygen Dissociation Rates—Oxygen dissociation from Hb I is about 5-fold faster than from mammalian myoglobin, but is characteristic of many clam tissue hemoglobins. It is about 500–800-fold faster than dissociation from Hb II and Hb III. The rates of oxygen dissociation were independent of pH from pH 5.2 to 9.0 (Hb I) or pH 5.9 to 9.0 (Hb II and Hb III). The rate of oxygen dissociation from an equimolar mixture of Hb II and Hb III corresponded to an average of the rates for the individual proteins. These rates are collected in Table IV.

Carbon Monoxide Combination Rates—The second order rates constant for combination of carbon monoxide with Hb I is close to that of myoglobin and 40–100-fold faster than those for Hb II and Hb III (Table IV).

Carbon Monoxide Dissociation Rates—The rates of dissociation of carbon monoxide from Hb I, Hb II, and Hb III and myoglobin are all similar (Table IV).

Oxygen Affinities —The oxygen affinity of Hb I, P50 = 0.2 Torr, at 20 °C was independent of pH from pH 6.4 to 8.4, and independent of hemoglobin concentration from 90 μM to 1 mM. The interaction coefficient of the Hill equation was unity throughout. The equilibrium oxygen affinities of Hb II and Hb III, 0.1 Torr in each case, were independent of protein concentration from 0.1 to 1.2 mM and 0.1 to 1.6 mM, respectively. Oxygen affinity, K′, derived from the kinetic con-

\[ K' \text{ (mM) } \]

stants for oxygen combination and dissociation, determined at 1.5 μM final heme concentration where the proteins are monomeric, are the same as those determined at equilibrium using more concentrated solutions. Values are collected in Table IV. The apparent oxygen affinity of an equimolar mixture of Hb II and Hb III (1.4 mM total heme) was 0.1 Torr, with Hill's n = 1.1.

Oxidation/Reduction Potentials—The equilibria of the ferric/ferrous couples of Hb I, Hb II, and Hb III each fit the relation for a simple one-electron process, with an average limiting slope of 59 mV/10-fold change in the ratio ferric/ferrous hemoglobin at 20 °C (Fig. 5). The midpoint potential of Hb I is invariant from pH 6.4 to 9.0; E₀ = 103 ± 8 mV (Fig. 5A).

The pH dependence of the midpoint potential of Hb II fits the relation for a single ionization, slope 58 mV/pH, of pK 6.6 with limiting values of 146 ± 2 and 93 ± 8 mV at the acid and alkaline limits, respectively (Fig. 5B). Following the treatment of Dutton (50) we calculate values at pH 5.7 and 7.7 for the pK of the protonation of the oxidized and conjugated-reduced forms, respectively (Fig. 5B).

The pH dependence of the midpoint potential of Hb III approximates a linear relation with slope 55 mV/pH of pH 5.5 to 8.4, with a limiting value of 165 ± 5 mV at the acid extreme (Fig. 5A). The pK of protonation of the oxidized form calculated as before is approximately 5.6. Under the conditions of these measurements the midpoint potential of horse heart myoglobin, 70 ± 4 mV (Fig. 5D), was nearly independent of pH from pH 6.6 to 9.0, in agreement with earlier results (51).

Ferric Hemoglobin Sulfide—Ferric Lucina Hb I, Hb II, and Hb III each react with hydrogen sulfide to generate similar species whose optical spectra are characterized by absorption maxima at 425 and 545 nm with a conspicuous shoulder near 573 nm (Fig. 6). These optical spectra are sensibly the same as those of the product, ferric myoglobin or hemoglobin sulfide, generated by reaction of ferric whale myoglobin and sulfide, or ferric human hemoglobin and sulfide (22). Electron paramagnetic resonance spectra confirm the identity of these products as ferric hemoglobin sulfide (52). Ferric hemoglobin may be regenerated by diluting the solutions (say 500-fold) so that the concentration of free sulfide is made small.

Equivalence of Heme and Bound Sulfide—Alkaline denaturation of ferric hemoglobin sulfide formed from each of the three hemoglobins and from whale myoglobin liberates 1.0 ± 0.1 g equivalent of sulfide/mol heme.

Formation of Ferric Hemoglobin Sulfide from Oxyhemoglobin—Lucina Hb I, when exposed to hydrogen sulfide (0.2 Torr) at an oxygen pressure (2 Torr) just low enough to partially desaturate the hemoglobin, forms the spectral entity identified as ferric hemoglobin sulfide with t₁/₂ of 350–1000 s (Fig. 7). The rate of ferric hemoglobin sulfide formation increased with increasing partial pressures of hydrogen sulfide. In contrast, Hb II and Hb III remained oxygenated under these conditions even when the hydrogen sulfide pressure is increased 10-fold to 2.0 Torr.

Sulphhemoglobin Is Not Formed—Sulphhemoglobin is not formed from Lucina Hb II, Hb III, or Hb III in the presence of hydrogen sulfide at low P0₂. Under these conditions, whale myoglobin and human hemoglobin are converted quantitatively to the corresponding sulfo-derivatives. Sulphhemoglobin is easily recognized by its green color and by a conspicuous, cyanide-insensitive, 620 nm absorbance maximum (53).

Reduction of Ferric Hemoglobin—Ferric Hb I is reduced slowly to ferrous hemoglobin by excess hydrogen sulfide. If a stream of oxygen is used to remove hydrogen sulfide from a

\[ K' \text{ (mM) } \]

\[ 1.0 ± 0.1 \text{ g equivalent of sulfide/mol heme.} \]

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Lucina Hemoglobin Kinetics

Table IV

Kinetic and equilibrium constants for the reactions of ferrous Lucina hemoglobins with oxygen and carbon monoxide compared to those of other proteins

| Protein          | \( k_a \times 10^4 \) M\(^{-1}\) s\(^{-1}\) | \( k_r \) \( \text{nm} \) | \( K' \) (M/\( \text{nm} \)) | \( P_50 \) \( \text{torr} \) | \( L_0 \times 10^4 \) M\(^{-1}\) s\(^{-1}\) | References |
|------------------|---------------------------------|----------------|----------------|----------------|---------------------------------|------------|
| Hb I             | 100–200\(^a\)                  | 61.1           | 0.18           | 0.780          | 0.0142                          | 18.2       |
| Hb II            | 0.390                          | 0.11           | 0.13           | 0.0190         | 0.0071                          | 370        |
| Hb III           | 0.388                          | 0.076          | 0.11           | 0.0072         | 0.0107                          | 1,465      |
| Whale Mb         | 19                             | 10             | 0.44\(^b\)     | 0.0185\(^b\)   | 42                              | 37         |
| Soybean Lb       | 116                            | 1.2\(^b\), 5.55\(^b\) | 48             | 6.77           | 15                              | 58         |
| Bussumon Mb      | 47.3                           | 71             | 1,500          | 0.35           | 0.0062                          | 15         |
| Mb (His-64 → Gly)| 140                            | 1,600          | 11,400         | 5.8            | 0.038                           | 6.6        |
| Aplysia Mb       | 15                             | 70             | 4,667          | 0.5            | 0.02                           | 40         |
| Chironomus Hb\(^b\)| 300                        | 218            | 130            | 0.36           | 0.098                          | 35         |
| Diereocoeleum Hb\(^b\)| 300                 | 10\(^d\), 30\(^d\) | 33\(^d\), 100\(^d\) | 0.02\(^d\), 0.15\(^d\) | 110                             | 2.5\(^d\), 5.9\(^d\) |
| Glycera\(^c\)    |                                |                |                |                |                                 |            |
| Hb A             | 39                             | 385            | 9,800          | 2.15           | 0.022                          | 10.2       |
| Hb B             | 186                            | 1,800          | 9,700          | 26.8           | 0.042                          | 1.57       |
| Hb C             | 190                            | 2,800          | 14,700         | 22.4           | 0.055                          | 2.46       |
| Ascaris Mb       | 12                             | 0.23           | 192            | 0.11           |                                 |            |

\(^a\) Estimated from \( k_{tof} \) and \( P_{so} \).
\(^b\) This study.
\(^c\) \( P_{so} \) calculated from \( K' \) is 0.16 torr for Hb II and 0.14 for Hb III.
\(^d\) Acid limit.
\(^e\) Alkaline limit.
\(^f\) Distal residue is valine (46).
\(^g\) Distal residue is isoleucine (47).
\(^h\) Distal residue is tyrosine (48).
\(^i\) Distal residue is leucine (49).

The second order rate constants for the reduction of aquo-ferric Hb I and ferric Hb II by dithionite were \( 2.9 \times 10^4 \) and \( 2.1 \times 10^4 \) M\(^{-1}\) s\(^{-1}\), respectively. The reduction of ferric hemoglobin sulfide proceeded nearly isobestically (547, 578 nm) with no suggestion of a long-lived ferrous hemoglobin sulfide primary product. The range of dithionite concentrations used was too small to distinguish whether \( \text{S}_2\text{O}_5^- \) or SO\(_2\) was the kinetically important reducing species (54). The results of Lambeth and Palmer (54) and of Olivas et al. (55) on reduction of ferric myoglobin and several liganded ferric myoglobin species suggests that SO\(_2\) is the most probable reducing species. At a dithionite concentration of 1 mM the reduction of ferric Hb I sulfide to ferrous Hb I and hydrogen sulfide proceeds 10 times faster than dissociation of the ligated sulfide, described below.

**Reaction of Ferrous Hemoglobin with Ferricyanide**—Second order rate constants for oxidation of ferrous to ferric Hb I, Hb II, Hb III, and myoglobin were: \( 0.74 \times 10^6 \), \( 0.12 \times 10^6 \), \( 0.04 \times 10^6 \), and \( 1.74 \times 10^6 \) M\(^{-1}\) s\(^{-1}\), respectively, at pH 7.5 and 20 °C. The values for Mb are in close agreement with those reported earlier (56).

**Rates of Combination with Hydrogen Sulfide and Methanethiol**—The rate of combination of ferrous Hb I with hydrogen sulfide increased 3000-fold from \( K' = 66 \times 10^2 \) s\(^{-1}\) at the alkaline limit, pH 10.5, to \( k' = 224 \times 10^2 \) M\(^{-1}\) s\(^{-1}\) at the acid limit, pH 5.5. The pH dependence of the rate follows the relation for a single ionization of pK 7.0 (Fig. 8A). The pH dependence of the rate follows the relation for a single ionization of pK 7.0 (Fig. 8A). The rate of combination of Hb II with methanethiol increases 8-fold from \( k' = 134 \times 10^2 \) M\(^{-1}\) s\(^{-1}\) at the alkaline limit, pH 10.5, to \( k' = 1080 \times 10^2 \) M\(^{-1}\) s\(^{-1}\) at the acid limit, pH 7.5. The pH dependence of the rate follows the relation for a single ionization of pK = 9.4. The combination of ferric Hb I with hydrogen sulfide or methanethiol is single kinetic events, homogeneous and first order to greater than 90% completion at all pH (Fig. 9A). The rates increase linearly as functions of ligand concentration showing that the reactions obey second order kinetics at each pH (Fig. 9B).

The rates of combination of ferric Hbs II and III with hydrogen sulfide increase markedly between pH 9.0 and 5.5. The rates of the reaction of Hb II reach limiting values of 32 torr at pH 7.5 and 20 °C. The values for Mb are in close agreement with those reported earlier (56).

**Fig. 6.** Optical spectra of ferric Lucina Hb I and Hb II sulfides formed by reaction of the ferric Hbs with a slight excess of Na\(_2\)S in 0.05 M phosphate buffer, pH 7.5. Solid line, Hb I; dashed line, Hb II. Ferric Hb III sulfide exhibits a spectrum nearly identical to that of Hb II.
were in reasonable agreement with those calculated from the precision of equilibrium measurements with Hb I. The binding constant learned from the ratio of the rate constants (Table V) with pK 6.7 for the conversion (Table VI). At pH above 7.0 the reaction is best fitted by an ionization value greater than 1; below pH 7.0 it is best fitted by a value of 2.0 (Fig. 8F). A single kinetic event, homogeneous and first order to greater than 90% completion was observed at all pH (Fig. 10A). The rates increased linearly as functions of ligand concentration showing that the reaction obeys second order kinetics at each pH from 8.5 to 5.5, (Fig. 10B), with small deviations at higher ligand concentration.

The progress of the reaction of ferric Hb III with hydrogen sulfide is complex. At pH 8.4, the alkaline limit of spectral change, the progress curve can be resolved into at least two dominant simultaneous rates: 4.0 M⁻¹ s⁻¹ (approximately three-fourths of the absorbance change at 436 nm) and 120 M⁻¹ s⁻¹ (approximately one-fourth of the absorbance change) (Table V). At pH 5.5, near the pK for change from alkaline to acid ferric species (52), the reaction can be resolved into at least two simultaneous rates: k' = 9.200 M⁻¹ s⁻¹ (one-third of the absorbance change) and k' = 38,000 M⁻¹ s⁻¹ (two-thirds at the absorbance change). Each of these rates increases linearly with hydrogen sulfide concentration. Although the faster rate at pH 8.4 is clearly contaminated by an additional event. At an intermediate pH, pH 6.5, the reaction could be resolved into three simultaneous rates, k' = 120, k' = 1,900, k' = 10,600 M⁻¹ s⁻¹. The pH dependence of the rate constants of each of these three reactions approximates the relation for a single ionization (Fig. 8C).

Dissoociation of Hydrogen Sulfide—Dissoociation of hydrogen sulfide from ferric Hb I sulfide is extraordinarily slow, k = 2.2 × 10⁻⁴ s⁻¹. This is 75-fold slower than dissoociation from Hb II and Hb III and 200-fold slower than dissoociation from myoglobin (58) (Table V).

Equilibrium Binding of Hydrogen Sulfide—The equilibrium binding constants learned from equilibrium measurements were in reasonable agreement with those calculated from the ratio of the rate constants (Table V). The high sulfide affinity of Hb I and the large solubility of hydrogen sulfide limited the precision of equilibrium measurements with Hb I. The binding constant learned from the ratio of the rate constants is considered the more reliable value. The affinity of ferric Hb I for hydrogen sulfide (K' = 3.4 nM) is 4000-fold greater than those of ferric Hb II, Hb III, and whale myoglobin.

**DISCUSSION**

Hemoglobin, often present in the host tissue of molluse/bacteria symbioses (6), reaches the extraordinary concentration of 1.5 mM in the bacteria-housing gills of the clam _L. poecinata_. The gills appear nearly black by virtue of the cytoplasmic hemoglobin. Three hemoglobin monomers, together with an abundant protein lacking obvious prosthetic groups, comprise the largest part of the soluble protein which may be extracted from the gill. The hemoglobins are each present at approximately 0.5 mmol (heme) kg⁻¹ wet weight tissue. Following Read (15, 16), who first called attention to these hemoglobins, they may be called Hb I, Hb II, and Hb III, after the order in which they emerge from a DEAE column. The amino acid composition of Hb I is distinct from those of Hb II and III which are very similar one to another. In addition a cystein-rich protein not encountered in other hemoglobin-containing tissues is present at a concentration of 0.66 mmol kg⁻¹ wet weight tissue.

Isolated Hb I is monomeric under all conditions (Table I). Hemoglobin II, monomeric in dilute solution, tends toward higher oligomers in concentrated solution; Hb III tends toward dimers, and an equimolar mixture of Hb II and Hb III is tetrameric when concentrated. This suggests that the tetramer, (Hb II₄ (Hb III)₄, may exist in the tissue. If so, it is a noninteractive tetramer, because Hill's n is always unity and the oxygen affinities of each hemoglobin and of the mixture are independent of protein concentration.

The molecular weight of tissue hemoglobins, narrowly distributed near 17,000–18,000, approximately 150 residues, might suggest a lower size limit for a protein capable of binding oxygen reversibly. The constant pattern of introns in many hemoglobin genes has suggested the hypothesis that the central peptide, residues 31–104 of myoglobin, encoded by the central exon of the myoglobin gene may be sufficient for reversible oxygen binding (59). Additional conformational stability may be provided by the first and/or third exons encoded sequences. A somewhat larger heme-peptide "minimyoglobin," residues 32–139, in fact binds oxygen reversibly with rates of combination and dissoociation similar to those of native myoglobin (59). Hemoglobin I, with 134 residues, may approach the lower limit defined by minimyoglobin with 108 residues.

Reactions with Oxygen—The equilibrium oxygen affinities of the three purified hemoglobins are all approximately the same, P_O₂ = 0.1–0.2 Torr (Table IV), in agreement with the value reported earlier for total hemoglobin in a tissue extract (16). They fall about mid range of oxygen affinities reported for diverse invertebrate tissue hemoglobins (60). These similar...
oxygen affinities of the three Lucina hemoglobins are achieved by very different balances of combination and dissociation rates.

The estimated rate of combination of Hb I with oxygen is conspicuously fast, rivalling the fastest reported oxygen combination rates (Table IV). Several hemoglobins which lack distal histidines combine rapidly with oxygen (Table IV). This raises the possibility that Hb I may lack a distal histidine residue. In contrast, the rates of combination of Hb II and Hb III with oxygen are about 3 orders of magnitude less than that of Hb I and are among the slowest known (Table IV). Carbon monoxide combination with Hb II and Hb III is likewise 40–100-fold slower than combination with Hb I or with myoglobin. This suggests that some special configuration of a distal ligand or distal heme pocket may stabilize deoxy Hb II and III or impede access of a ligand approaching the heme iron atom.

Overall rates of reactions of hemoglobins with oxygen and carbon monoxide have been analyzed in terms of a rate of reaction at the heme and rates of diffusion between the protein internal and external environments (61, 62). The rates of dissociation of carbon monoxide from Lucina Hbs I, II, and III, as well as from diverse other monomeric hemoglobins are all nearly the same, differing one from another for the most part by no more than a factor of 4 (Table IV). This suggests that constraint on diffusion of ligands away from the immediate vicinity of the heme does not dominate the measured overall rate of ligand dissociation. In contrast to the results with carbon monoxide, dissociation of oxygen from Hb II and Hb III is nearly 3 orders of magnitude slower than dissociation from Hb I and is very slow compared with rates of dissociation of oxygen from many other hemoglobins (Table IV). This implies stabilization of the oxygengated structure. Residues in the distal heme pocket have been implicated in control of oxygen dissociation rates; the distal histidine in particular playing a major role in many proteins (30, 61, 62). Hydrogen bond formation between the distal histidine and the bound oxygen molecule stabilizes bound oxygen and slows its dissociation from the protein (30, 39, 61, 65–65). Oxygen dissociation from hemoglobins lacking this residue, Aplysia myoglobin (46), Chironomus hemoglobin (47), Dicrocoelium hemoglobin (48), the monomeric Glycera hemoglobins (49), and mammalian myoglobin (39) or hemoglobin (66) in which the distal histidine has been substituted by site-specific mutagenesis, tends to be fast (Table IV), and the possibility again arises that a distal histidine residue may be lacking in Lucina Hb I. Lucina Hb I does not meet the spectral criteria proposed by Shikama and Matsuoka (67) to identify hemoglobins lacking a distal histidyl residue. However, spectra of each hemoglobin may be influenced by the nature of the residue replacing histidyl, and it is not clear that their criteria can be extended to cover the full diversity of such proteins. Certainly their criteria do not apply to Dicrocoelium hemoglobin, those Hemoglobins M or Lucina Hb II and III in which interaction between distal tyrosine and the heme may strongly influence the optical spectrum.

The extreme slow rates of dissociation of oxygen from ferric Lucina Hb II and Hb III suggests that an unidentified distal residue may interact strongly with the bound oxygen molecule to slow its rate of dissociation. Elsewhere, we suggest that a tyrosyl residue in the heme pocket may ligate to the ferric heme iron (52), as a tyrosinate does in ferric Dicrocoelium hemoglobin (48). The distal residue of Dicrocoelium hemoglobin is believed to change its orientation (perhaps be expelled from the heme pocket) upon reduction of the heme iron, leaving the heme accessible to rapid combination and dissociation of ligands (48) (Table IV). In contrast, we suggest that a tyrosyl (or nearby residue) may remain in the heme pocket of ferrous Hb II and III where it may interact with the bound oxygen molecule and contribute to the very slow rates of oxygen dissociation from these proteins.

Reactions with Sulfide—We now direct attention to the ferric forms of Lucina hemoglobins, and from their properties attempt to deduce how these cytoplasmic hemoglobins may function in the clam/bacterial symbioses. We begin by identifying that hemoglobin which reacts preferentially with sulfide by exposing each of the three hemoglobins isolated from the gill of the clam, L. pectinata, to hydrogen sulfide (0.2–2.0 Torr) at a PO, (~2 Torr) just sufficient to largely saturate the hemoglobin. Only Hb I is converted to ferric hemoglobin sulfide. The identity of the product, ferric hemoglobin sulfide, is established by similarity of optical (Fig. 6) and EPR spectra (52) to those of authentic ferric myoglobin sulfide (22, 53) and by the composition of the product which contains one bound sulfide/heme. Hb II and Hb III remain oxygenated under all conditions examined. Sulfhemoglobin formation is never observed.

Ferric Hemoglobin Sulfide Formation from Oxyhemoglobin—Oxy-Hb I reacts with hydrogen sulfide to form ferric hemoglobin sulfide at a rate which is only somewhat slower than the rate of the same reaction observed in intact gill filaments of another species of clam (14). The kinetics of the reaction are complex, in part because of competing reactions such as reduction of the product by hydrogen sulfide. Hemoglobin I at low oxygen pressure (PO, ~ 2 Torr), where perhaps 5% of the hemoglobin is deoxygenated, is stable in the absence of hydrogen sulfide. Ferrous Hb I is stable in the presence of hydrogen sulfide. The relatively rapid reaction (ts ~ 350–1000 s) in the simultaneous presence of oxygen and hydrogen sulfide suggests that the reaction may proceed by nucleophilic displacement (68, 69) of bound superoxide anion from oxyhemoglobin, Hb(heme d5J .OO-, by the hydrosulfide anion, HS-. If so, the reaction between oxy Hb I and sulfide is three orders of magnitude more rapid than comparable reactions such as the formation of ferric myoglobin azide by nucleophilic attack of azide anion on oxymyoglobin (69).

The more rapid reaction of Hb I cannot be a consequence of a difference in oxidation/reduction potentials, because the potentials of Hb I, Hb II and Hb III are all very similar at neutral pH.

Combination of Ferric Hb I with Sulfide—The second order rate constant for combination of ferric Hb I with hydrogen sulfide increases roughly 3 orders of magnitude from pH 10.5 to 5.5, with apparent pK = 7.0. This apparent pK is coincident with that for ionization of hydrogen sulfide (pK = 7.0) (70). Methanethiol, CH3SH, which is protonated at all pH in the range considered (pK > 10.3, Refs. 71 and 72), reacts with Hb I near neutral pH, at a rate (k' = 10.8 × 109 M−1 s−1) comparable to the rate of reaction of Hb I with hydrogen sulfide at the acid limit (k' = 2.24 × 109 M−1 s−1). This suggests that undissociated hydrogen sulfide is the attacking species, and that the largest part of the change in rate near neutrality may be ascribed to protonation of HS− to H2S.

The pH dependence of the rate of methanethiol binding exhibits a single inflection with pK 9.4 (Table VI). The transition from hydroxyferric to aquoferric Hb I also occurs in this range (pK = 6.6) (52). The simplest explanation is that aquoferric Hb I reacts more rapidly with methanethiol than does the alkaline hydroxyferric form. Rapid combination of ferric Hb I with ligands is particular to hydrogen sulfide or methanethiol. Combination of hydrogen cyanide with Hb I
proceeds at about the same rate as combination with myoglobin (73).

Combination of Ferric Hb II and Hb III with Sulfide—
Solutions of ferric Hb II and Hb III contain three species. At acid pH, aquoferric hemoglobin predominates. At alkaline pH, two species coexist in constant proportion, each contributing about half of the mixture. These species are hydroxyoxygen may be the distal ligand to the heme (52). The pH dependence of combination of Hb II with hydrogen sulfide fits the relation for more than one ionization. The apparent pK (pK = 6.7), is coincident with the pK (pK = 6.6) for the acid/alkaline transition (Table VI). Protonation of HS− to
H2S and of an ionizable group on at least one of the three ferric species may contribute to the observed change. The kinetics of reaction at every pH are consistent with a single bimolecular reaction whose rate is proportional to hydrogen sulfide concentration. This suggests that the rapid rate of combination with sulfide at the acid limit is a measure of the rate of reaction of aquoferric Hb II with hydrogen sulfide, and that rates at higher pH values are proportional to the fraction of aquoferric Hb in the mixture. This requires that the rate of conversion of alkaline species to aquoferric hemoglobin be faster than the rate of reaction with hydrogen sulfide. A rough estimate of the rate of conversion of alkaline to acid forms of Hb II was obtained from a few stopped flow pH-jump experiments, from pH 7.5 to 5.5, followed at 403 and 421 nm. The rates of alkaline/acid conversion were roughly 10-fold greater than the measured rate of combination with sulfide at the relatively low sulfide concentrations used (60–480 μM). We suggest that the reaction of ferric Hb II with hydrogen sulfide proceeds predominantly through a common intermediate, aquoferric hemoglobin.

Reaction of Hb III follows a different course. The progress of the reaction of ferric Hb III with hydrogen sulfide, at any pH from pH 7.5 to 5.0 may be resolved into three widely divergent simultaneous rates. It is reasonable to presume that these reflect separate reactions of each of the three species present in solutions of ferric Hb III.

Affinities of Lucina Hemoglobin for Hydrogen Sulfide—Near neutral pH, the hydrogen sulfide affinity of ferric Hb I is more than three orders of magnitude greater than the affinities of Hb II, Hb III or myoglobin. Table V presents equilibrium affinities for hydrogen sulfide expressed as K′ and Keq at pH 7.5. The great affinity of Hb I is achieved by relatively rapid combination together with very slow dissociation. The rate of hydrogen sulfide combination with Hb I at this pH is 50–100-fold faster than combination with Hb II, Hb III, or myoglobin; the rate of dissociation about 75 times slower.

Reduction of Ferric Hemoglobin Sulfide—Reduction of ferric Hb I sulfide by dithionite (>5 mM) to ferrous Hb I and hydrogen sulfide proceeds roughly 50 times faster than dissociation of the ligated sulfide. It follows that ferric hemoglobin sulfide reacts directly with dithionite without prior dissociation of the ligand. The expected intermediate, ferrous hemoglobin sulfide, was not detected, suggesting that, if formed, it is short-lived. Direct reduction of ferric hemoglobin sulfide has precedents in the direct reduction of ferric hemoglobin or myoglobin cyanides (55, 74), ferric myoglobin imidazole (55, 74, 75) ferric cytochrome c cyanide (76), ferric leghemoglobin nitric oxide (77), oxyhoroerasid peroxidase (78), and cytochromes β and c, which have distal ligands at all times.

Conclusion—Conversion of oxyhemoglobin to ferric hemoglobin sulfide, observed in the living gill, has been reproduced using purified hemoglobin I. Hemoglobin I, the sulfide-reactive hemoglobin, is distinguished by this reactivity from hemoglobins II and III, oxygen reactive hemoglobins, which remain oxygenated. The outstanding property of hemoglobin I is the extraordinary affinity (3.4 μM) of the ferric hemoglobin for hydrogen sulfide, a consequence of rapid combination and very slow dissociation. We postulate that a major function of hemoglobin I is to deliver sulfide to the bacterial symbiont. It seems improbable that this could be achieved by simple dissociation of ligated sulfide because the slow rate of ligand dissociation implies a turnover time of about 5000 s. Instead, we suggest that ferric hemoglobin I sulfide may accept electrons in a rapid reaction to liberate the ferrous protein and hydrogen sulfide.

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Lucina Hemoglobin Kinetics

Materials and Methods

Lucina proteus - were collected from shallow bays near La Pargua, Puerto Rico. Enriched gills, about 4 g per animal, were frozen immediately in liquid nitrogen and were stored at -70 °C.

Lucina Hemoglobin - Hemoglobin extraction follows the general method of Schuder et al. [24] with modifications introduced by Appleby et al. [25]. Frozen gill, 25 g, were pulverized under liquid nitrogen in a porcelain mortar. Subsequent operations were performed at temperatures lower than 0 °C in an argon atmosphere. The powdered gill, still wet with liquid nitrogen to exclude air, was suspended in 100 ml of a carbonate buffer (pH 9.0), containing 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol. The suspension was sparged continuously with a stream of carbon dioxide and allowed to warm until thawed completely. The slurry, usually pH 6.8, was adjusted to pH 7.5 by addition of 1 M tris(hydroxymethyl)aminomethane buffer, pH 8.0. The mixture was then transferred to separate centrifuge tubes (Oak Ridge tubes, Nilagen, Rochester, N.Y.) and centrifuged at 12,000 g for 20 min. The dark red supernatant was clarified by vacuum filtration through a pad of "Cell Debris Remover" (Whatman, Clifton, N.J.) 0.5 x 8 cm, previously washed with carbon monoxide-saturated buffer. Part of the filtrate was transferred under carbon monoxide to a large syringe fitted with a long 16 gauge needle; the balance was frozen for storage at -70 °C.

Gel Filtration Chromatography - Portions, 30 ml, of the filtrate were applied to a column of Sephadex G-75 superfine (Pharmacia, Piscataway, N.J.) 5 x 50 cm. The effluent was collected at a flow rate of 2 ml/min with carbon monoxide-saturated 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA. The effluent fractions are stable at air.

The protein fractions were concentrated separately by ultrafiltration over Amicon YM-10 membranes (Amicon Corporation, Danvers, Mass.). The yellow, low molecular weight hemoglobin fraction was collected over an UM-2 concentration (in phosphate buffers) are stable to storage at liquid nitrogen temperature.

Ion Exchange Chromatography - Hemoglobin - Ion exchange chromatography was performed on a column of Sephadex G-25 superfine (Pharmacia, Piscataway, N.J.) 5 x 50 cm. The column was developed at a flow rate of 2 ml/min with carbon monoxide-saturated 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA. The effluent fractions are stable at air.

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Lucina Hemoglobin Kinetics

Hemoglobin of Lucina: Hemoglobin - A purified crystalline heme was determined by chromatography at 22 °C on a calibrated column of Sepharose 200 HR (Pharmacia). 1.0 x 30 cm, developed with 0.05 M potassium phosphate buffer containing 0.5 mM EDTA, pH 7.5, on a column of Superose-12 HR (Pharmacia) 1.0 x 30 cm. Calibration proteins were cytochrome c (molecular weight 12,000), bovine serum albumin (BSA) (67,000), and myoglobin (17,000) and the column was calibrated with a low-pressure microbore system (Pharmacia). The heme content of the sample was determined as the pyridine hemochromogen, assuming one heme per hemoglobin subunit. Hemoglobin amino acid content was expressed as moles of amino acid per mole of protein.

Apo-hemoglobin was prepared by extraction of the heme with methyl ethyl ketone (24.4) and the heme-free protein was isolated and characterized. The heme-free protein was found to be a monomeric protein with a molecular weight of 16,000. The heme-free protein was dissolved in buffer and the absorbance at 280 nm was measured.

Optical Spectra - The optical spectra of the heme-free protein were recorded using a Cary model 17 recording spectrophotometer. The spectra were recorded in the presence of buffer (0.05 M potassium phosphate, pH 7.5) and in the absence of buffer. The spectra were recorded at 20 °C and 4 °C.

Oxygen Equilibrium - These data were determined for a thromboplastin layer cell model derived from the design of Fink (19) and expressed in terms of oxygen saturation and Hill coefficients. The Hill coefficients were calculated from the oxygen saturation data using the Hill equation.

Carbon Dioxide Equilibrium - Equilibria with hemoglobins II and III were determined using the thin layer cell. The thin layer cell was used to determine the oxygen saturation and carbon dioxide equilibrium of the heme-free protein. The thin layer cell was a glass cuvette with a thickness of 1.0 mm. The heme-free protein was dissolved in buffer and the cuvette was placed in an oxygen equilibration chamber. The oxygen saturation and carbon dioxide equilibrium were determined using the thin layer cell.

Sulfide - Small volumes (5 μl) of solutions of ferric hemeoglobin were added to a stream of oxygen-free nitrogen and the absorbance at 280 nm was measured. The absorbance at 280 nm was used to determine the oxygen saturation and carbon dioxide equilibrium.

Dissociation Rates - Solutions of oxy-hemoglobin were prepared using the thin layer cell. The solutions were mixed with solutions of deoxy-hemoglobin and the absorbance at 280 nm was measured. The absorbance at 280 nm was used to determine the oxygen saturation and carbon dioxide equilibrium.

Figures 1 and 2 show the oxygen and carbon dioxide equilibrium data for hemoglobins I, II, and III. The Hill coefficients were determined using the Hill equation.

Conclusion - The heme-free protein of Lucina hemoglobin has a molecular weight of 16,000 and is a monomeric protein. The heme-free protein has a Hill coefficient of 1.5 and a Scatchard plot with a slope of 1.5. The heme-free protein has a Hill coefficient of 1.5 and a Scatchard plot with a slope of 1.5.

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Figure 2. A) Elution profile of the combined low molecular hemoglobin and colorless cysteine-rich protein fractions developed on a column of DEAE Sepharose CL-6B. Ferric Hb II, myoglobin and a cysteine-rich protein are resolved. B) Elution profile of the light molecular weight hemoglobin fraction developed on a column of DEAE Sepharose CL-6B. OxyHb II and oxyHb III are resolved. Solid circles, absorbance at 540 nm; ordinate scale expanded 3-fold; open circles, absorbance at 280 nm; open triangles, sodium chloride concentration.

Table II. Amino acid composition of Lucina hemoglobins and the cysteine-rich protein given as moles of amino acid per mole of protein.

| Amino acid | Hb II | Hb III | Cysteine-rich protein |
|------------|-------|--------|-----------------------|
| Asp        | 13.9±0.4 | 14.6±0.1 | 17.2±0.6 |
| Glu        | 9.8±0.1  | 10.3±0.3 | 10.0±0.2 |
| Ser        | 9.4±0.1  | 9.3±0.1  | 9.1±0.1  |
| His        | 3.2±0.1  | 3.2±0.2  | 3.1±0.1  |
| Gly        | 15.4±0.7 | 15.9±0.1 | 16.0±0.1 |
| Thr        | 5.7±0.2  | 6.0±0.3  | 6.0±0.2  |
| Ala        | 15.0±0.1 | 15.5±0.1 | 15.5±0.1 |
| Val        | 6.4±0.2  | 6.0±0.1  | 6.1±0.1  |
| Asn        | 4.5±0.3  | 4.7±0.2  | 4.7±0.2  |
| Pro        | 2.9±0.2  | 3.5±0.1  | 3.5±0.1  |
| Phe        | 7.9±0.1  | 8.0±0.2  | 8.0±0.2  |
| Lys        | 8.5±0.1  | 9.0±0.2  | 9.1±0.2  |
| Arg        | 11.1±0.4 | 11.4±0.3 | 11.3±0.3 |
| His        | 5.1±0.1  | 5.1±0.1  | 5.1±0.1  |
| Tyr        | 1.3±0.1  | 1.8±0.2  | 2.1±0.2  |
| Cys        | 2.7±0.3  | 3.1±0.1  | 3.1±0.1  |

Table III. Spectral properties of Lucina hemoglobins.

| Hemoglobin | Hb I | Hb II | Hb III |
|------------|------|-------|--------|
| OD (540 nm) | 560 | 450 | 360 |
| pH 5.6 | 0.8 | 0.6 | 0.4 |
| pH 7.0 | 0.6 | 0.4 | 0.2 |
| pH 8.0 | 0.4 | 0.3 | 0.2 |

Figure 4. Optical spectra of Lucina Hemoglobin I. Solid line, oxyhemoglobin; dashed line, deoxyhemoglobin; dotted line, carbon monoxide hemoglobin. The ordinate scale for the visible region is expanded 10-fold.

Figure 5. Midpoint oxidation/reduction potentials of Lucina hemoglobins as functions of pH compared to that of myoglobin. A) Solid circles, Hb I and solid squares, Hb III. The midpoint potential of Hb I is independent of pH. The pH dependence of the midpoint potential of Hb III is approximately linear with a slope of 65 mV/pH unit from pH 5.5 to pH 8.4. B) Solid circles, Hb II and open squares, Hb III. The pH dependence of the midpoint potential of Hb II fits the relation for single titration of pH 6.0 with limiting values of 146 ± 2 mV at pH 5.6 and 93 ± 8 mV at the acid and alkaline limits respectively (Dole). Oxidation/reduction equilibria of Lucina Hb II. In each case the average limiting slope is 59 mV per 10 pH unit at the rate hemoglobinase hemoglobin. The midpoint potentials of Hb II are 149, 118, 85 mV at pH 5.7, 6.7 and 7.4 respectively.
Figure 7. Optical spectra of Lucina oxyHb I during the conversion to ferric Hb sulfide. PO2 = 1.9 torr, PH2S = 0.2 torr, pH 7.5 and 20 °C. Spectra were recorded at 0, 10, 32 min. The final ferric Hb sulfide spectrum was recorded after 60 min.

Figure 9. A) Progress of the reaction of ferric Hb I with sulfide at several pH. Heme 1.5 µM, sulfide 250 µM, after mixing. The reaction follows homogeneous pseudo-first order kinetics to 95% completion at each pH. B) Rate of reaction of ferric Hb I with sulfide as a function of sulfide concentration at 37 °C.

Figure 8. Second order rate constants for the reaction of ferric Lucina Hbs with sulfide as a function of pH. A) Hb I, Hb II and the most rapid reaction of Hb III. The lines are least squares fits of the Henderson-Hasselbalch equation for ionizations of n=1, n=2 and n=1.7 respectively. Three values for Hb are represented by X's. B) Hb II, the Henderson equations are n=1 and n=2, the pH in each case is 6.7. C) Three separate reactions of Hb III with fitted equations of n=1, n=1.2 and n=1.17 from slow to fast, the pH for each reaction is 6.1. The ordinate scale is adjusted to accommodate the different reactions.

Figure 10. A) Progress of the reaction of ferric Hb II with sulfide at several pH. Heme 1.5 µM, sulfide 200 µM, after mixing. The reaction follows homogeneous pseudo-first order kinetics to 95% completion at each pH. B) Rate of reaction of ferric Hb II with sulfide as a function of sulfide concentration at 37 °C.
Hemoglobins of the Lucina pectinata/bacteria symbiosis. I. Molecular properties, kinetics and equilibria of reactions with ligands.
D W Kraus and J B Wittenberg

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