THE STRUCTURE AND OXYGEN EQUILIBRIUM PROPERTIES OF THE INTRACELLULAR HEMOGLOBINS FROM THE BIVALVE MOLLUSC BARBATIA REEVEANA

by
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A THESIS

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The red blood cells of the bivalve mollusc Barbatia reeveana contain two hemoglobins. One, a tetramer ($M_r = 60,000$), is typical of the other intracellular hemoglobins. The other is unique among any intracellular hemoglobin studied so far. It is very large with a molecular weight of 430,000, and has a subunit with a molecular weight of 32,000 consisting of two oxygen binding domains. The structure and oxygen equilibrium properties of these intracellular hemoglobins are presented in this thesis.
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INTRODUCTION

Hemoglobin is the most ubiquitous respiratory protein found in vertebrate and invertebrate species (Prosser, 1973). Hemoglobin molecules reversibly bind oxygen and serve primarily to transport oxygen from the external environment to the organism's respiring tissues. Tissue hemoglobin, or myoglobin, facilitates diffusion of oxygen into tissues and may also store oxygen for use during periods of hypoxia (Scholander, 1960; Hemmingsen, 1963; Wittenberg, 1970).

The structure of vertebrate hemoglobin has been the subject of numerous investigations. Kendrew (1963) showed by x-ray crystallographic studies that sperm whale myoglobin consists of a folded polypeptide chain of 153 amino acid residues containing a "pocket" for the heme prosthetic group. Perutz (1964) reported that the structure of vertebrate hemoglobin contains four polypeptide chains which each resemble the structure of vertebrate myoglobin, and that the heterotropic and homotropic properties of vertebrate hemoglobin are dependent on this quaternary structure.

The structure of invertebrate hemoglobins are less well known. Like the vertebrate pigments, many
invertebrate hemoglobins appear to be constructed of multiples of 17,000 daltons as hypothesized by Svedberg (Svedberg, 1933; Svedberg and Pederson, 1940). Study of the tertiary structure of an annelid (Glycera) monomeric hemoglobin showed the typical myoglobin-like fold characteristic of vertebrate hemoglobins (Padlan and Love, 1974). A few invertebrate hemoglobins, however, have remarkably different structures. These invertebrate hemoglobins are unusual in that they are polymeric proteins which have subunits constructed of a linear series of covalently linked oxygen binding domains (Naxman, 1975; Wood and Mosby, 1975; Terwilliger et al, 1976; Terwilliger and Terwilliger, 1977; Terwilliger et al, 1978; Terwilliger and Terwilliger, 1978a and 1978b; Dangott and Terwilliger, 1979). Bivalve molluscs are one group of invertebrates that possess both these unusual domain series hemoglobin structures as well as the more typical vertebrate-like hemoglobins.

Many bivalve molluscs possess hemoglobin or myoglobin as a respiratory protein. When the molecules are intracellular, they appear as monomeric molecules with one heme and a molecular weight of 15-17,000, or dimeric, or tetrameric aggregates of this monomer. The red color of adductor muscle in Mercenaria mercenaria is the result of monomeric myoglobin of 16-17,000 molecular
weight (Koppenheffer and Read, 1969). Gill tissue of *Phacoides pectinatus* contains hemoglobin of 14,500 molecular weight, as well as some tetrameric and possible octomeric aggregates (Read, 1965). Circulating cells of the arcid bivalve *Noetia ponderosa* have a dimeric hemoglobin of 34,000 molecular weight (Freadman and Mangum, 1975). The intracellular hemoglobins of *Anadara transversa* have molecular weights of 30,000 and 58,000 (Yang, 1974), similar to the intracellular hemoglobins of *Anadara broughtonii* (= *A. inflata*), which are putative dimers and tetramers with molecular weights of 34,000 and 68,000 (Saskawa and Satake, 1967). The dimer is composed of 15,500 molecular weight electrophoretically indistinguishable subunits; the tetramer can be resolved into two dissimilar polypeptide chains, designated α and β, similar to the vertebrate hemoglobins (Furuta et al, 1977).

The hemocoelic fluid of some clams contain large extracellular hemoglobins (Manwell, 1963). The extracellular hemoglobin of the clam *Cardita* is 12 x 10^6 molecular weight and contains one heme per 16-22,000 g protein (Waxman, 1975; Terwilliger et al, 1978). Upon denaturation of *Cardita borealis* extracellular hemoglobin, subunits of 290-300,000 molecular weight are produced; when digested with subtilisin this subunit yields
15-17,000 molecular weight fragments that reversibly bind oxygen (Terwilliger and Terwilliger, 1978a). Extracellular hemoglobins from the bivalves Astarte castanea and Cardita affinis are of similar construction (Terwilliger and Terwilliger, 1978b). A structure for these clam polymeric hemoglobins has been suggested similar to that proposed for the planorbid snail Helisoma hemoglobin (Terwilliger et al., 1976). These polymeric molluscan hemoglobins may be aggregations of subunits that consist of a covalently linked series of oxygen binding domains (Terwilliger et al., 1976; Terwilliger et al., 1978; Terwilliger and Terwilliger, 1978a and 1978b).

The structures of intracellular and extracellular clam hemoglobins appear to differ from one another strikingly. The low molecular weight bivalve myoglobins and hemoglobins are contained within cells, whereas the large clam hemoglobins, which consist of an aggregation of subunits composed of linked oxygen binding domains, appear to be extracellular.

Oxygen equilibrium studies have been carried out on myoglobin and hemoglobin from several bivalve species. Myoglobin of Mercenaria mercenaria exhibits high oxygen affinity ($P_{50} = 0.55 \text{ mm Hg}$) and shows no cooperativity or Bohr effect (Manwell, 1963). These characteristics
are shared by other molluscan monomeric myoglobins (Read, 1966), as well as by myoglobin from humans (Rossi-Fanelli and Antonini, 1958), horse (Theorell, 1934) and hagfish (Manwell, 1958).

Clam hemoglobins located within circulating cells have lower oxygen affinities than myoglobins. Djangmah et al (1978) found that for the arcid Anadara senilis, the dimeric hemoglobin has a $P_{50} = 4.2-6.7$ mm Hg and that the tetrameric hemoglobin has a $P_{50} = 8.0-13.0$ mm Hg; both show cooperativity and have small Bohr effects. Anadara satowi dimeric hemoglobin has a $P_{50} = 9.0$ mm Hg, shows no Bohr effect, and exhibits cooperativity with a Hill coefficient of $N = 1.5$; the tetramer has a $P_{50} = 11.0$ mm Hg and shows both a slight reverse Bohr effect and strong cooperativity with $N = 2.1$ (Ohnoki et al, 1975). Oxygen equilibrium studies on unseparated hemoglobin solutions from Anadara broughtonii indicate a $P_{50} = 10$ mm Hg with $N = 1.15$ at 20°C (Kawamoto, 1928). Both A. broughtonii dimeric and tetrameric hemoglobin components show strong heme-heme interactions with $N = 1.8$ and 2.8-3, respectively; however, with a change in pH there is no concomitant change in the oxygen binding properties of these hemoglobins (Furuta et al, 1977).

This lack of a Bohr effect for A. broughtonii hemoglobin is in marked contrast to the heterotropic
properties exhibited by vertebrate hemoglobins, particularly since both the tetrameric structure of this clam and vertebrates share a \( \alpha_2 \beta_2 \) structure. Perutz (1970) suggested a mechanism for the Bohr effect of human HbA hemoglobin requiring, in part, a critical role for the carboxy terminal histidine residues in the \( \beta \) chains. The lack of a Bohr effect for A. broughtonii tetrameric hemoglobin may be attributed to the substitution of leucine residues in place of histidine as the carboxy termini of the \( \alpha \) and \( \beta \) chains (Furuta et al, 1977).

Oxygen equilibrium studies by Manwell (1963) on hemoglobin from another arcid clam, Noetia ponderosa, show a \( P_{50} \) = 13.5 mm Hg and a Hill coefficient of \( N = 1.1 \) at 22-24 °C, with no Bohr effect in the pH range 6.68-8.24. Freadman and Mangum (1976) reported a \( P_{50} \) = 5.30 at 10 °C and 6.25 mm Hg at 23 °C for Noetia ponderosa hemoglobin within intact red cells in the pH range 6.6-7.4. These half-saturation values given by Freadman and Mangum (1976) are similar to the values given by Yang and Parkurst (1973) for Noetia ponderosa hemoglobin extracts. This similarity in oxygen affinity of hemoglobin in intact cells and hemoglobin extracts of Noetia ponderosa suggests that the oxygen binding properties of this hemoglobin are not modified by intracellular constituents (Freadman and Mangum, 1976). This same
finding has been reported for other invertebrate hemoglobin (Mangum and Carhart, 1972; Mangum et al, 1975).

Collett and O'Gower (1972) have reported the unusual temperature dependent oxygen binding behavior of hemoglobins from the red cells of Anadara granosa, Anadara maculosa, and Anadara trapezia. These authors found that the P₅₀ values for hemoglobin extracts from these three clams increases with temperature over the range 15-20 °C (at 20 °C the P₅₀ for A. granosa and A. maculosa is 5.6-5.9 mm Hg, that for A. trapezia is 10.5 mm Hg). Remarkably, however, at the higher temperature range (20-30 °C), there is an increase in oxygen affinity with increasing temperature. This unusual sinistral shift in the oxygen equilibrium curve at high temperatures may serve as a physiological adaptation to the decreased availability of oxygen in warm water (Collett and O'Gower, 1972).

The functions of large extracellular clam hemoglobins have not been as extensively characterized as the other bivalve pigments. The extracellular hemoglobin of Cardita floridana at 22-24 °C has a P₅₀ value of 11 mm Hg, independent of pH in the range 7.03-7.5, and a Hill coefficient slightly less than 1 (Hanwell, 1963). The lack of any homotropic or heterotropic interactions for this large complex molecule is vexing. The 16,000 dalton
heme-containing domains generated by enzymatic digestion share the same oxygen binding properties of the intact pigment (Terwilliger and Terwilliger, 1978a). The oxygen affinities of clam circulatory hemoglobins, both within cells and extracellular (in the range 10 - 20 mm Hg) are lower than most other molluscan hemoglobins (Read, 1966), although not as low as some vertebrate hemoglobins (human HbA has a P50 = 26 mm Hg under physiological conditions) (Manwell, 1963).

The purpose of this thesis is to examine the structure and function of the hemoglobins from the bivalve mollusc Barbatia reeveana. I collected this arcid from the wave-exposed intertidal rocks in the Gulf of California and noticed that they contained abundant hemoglobin which seemed to be intracellular. As this species was previously unstudied and since it was found in a semi-tropical environment, its respiratory physiology might be interesting, especially in light of the unusual temperature effects exhibited by the arcid clams studied by Collett and O'Gower (1972). One of the most remarkable findings of this study discussed below is the occurrence and structure of a polymeric (Mr = 430,000) hemoglobin contained within cells, as well as a smaller, more typical tetrameric pigment. The structural and functional characteristics of these hemoglobins were
studied and the results are presented in this thesis.
MATERIALS AND METHODS

Barbatia reeveana (Orbigney) was collected from the wave-exposed rocky intertidal, near El Coyote, in Conception Bay, Baja California, Mexico, and identified according to Keen (1971). The animals were transported to the laboratory where they were kept in aerated aquaria at room temperature (19-23 °C) with daily fresh change of seawater (32°/oo).

Blood was collected by inserting a pipette into the clam's pallial sinus. Blood from 1-5 animals was pooled and washed three times with ice-cold 3 % NaCl. The final cell pellet was resuspended in 1 mM Tris·HCl (pH 8.0) and placed on ice for 1 hr. The resulting solution was centrifuged at 12,000 x g for 10 min. and the supernatant was applied to a column (1.9 x 100 cm) of Sephadex G-100 equilibrated with 0.05 M Tris·HCl buffer (pH 8.0) 0.1M in NaCl. The column was previously calibrated with Blue Dextran, bovine serum albumin, ovalbumin, α-chymotrypsinogen A, and sperm whale myoglobin (Sigma Chem. Co.).

Hematocrit values were obtained from blood freshly collected and immediately taken up in a micro-hematocrit capillary tube and centrifuged at 3,000 rpm until no
further packing of the cells occurred (5 min.). The blood hemoglobin concentration was determined by converting the pigment in the cell lysate to the cyanmethemoglobin E = 11.0 (van Assendelf, 1975). A heme to protein ratio of 1:16,000 was used in calculating hemoglobin concentrations.

The Hb-H hemoglobin component was concentrated by vacuum dialysis and dialyzed versus either 0.05 I Tris·HCl (pH 8.0) 0.1 M in NaCl or, 0.05 I Tris·HCl (pH 7.0) 0.1 M in NaCl, and 0.01 M in MgCl₂. The hemoglobin was applied to a column (1.9 x 100 cm) of Bio-gel A-5M, 200-400 mesh, (Bio-Rad Lab.) previously equilibrated with either of the above buffers. The Bio-gel column was calibrated with Eudistyliya vancouverii chlorocruorin (Mr = 2.8 x 10⁶) (Terwilliger et al, 1975), Cancer magister hemocyanin (Mr = 9.0 x 10⁵ and 4.5 x 10⁵) (Ellerton et al, 1970) and bovine heart lactate dehydrogenase (Mr = 150,000) (Sigma Chem. Co.).

Sedimentation velocity experiments were carried out on hemoglobin in 0.05 I Tris·HCl (pH 8.0) 0.1 M in NaCl with a Beckman-Spinco Model E ultracentrifuge equipped with a RTIC temperature control unit and scanning optics. The temperature was near 20 °C and the rotor speeds were 36,000 rpm for the 13.6 S material
(1.5 mg/ml) and 44,000 rpm for the 9.7 S material (0.7 mg/ml). Sedimentation coefficients were calculated by the method of least squares from the plot of $\ln r$ vs. $s$ and corrected to the viscosity and density of water at standard conditions (Svedberg and Pederson, 1940).

The Hb-H hemoglobin was dialyzed against 0.01 M ammonium bicarbonate and applied to a column (1.9 x 12 cm) of DEAE (diethylaminoethyl)-cellulose (Sigma Chem. Co.) equilibrated with the same buffer. The column was washed with a large volume of this buffer, and the pigment was eluted by establishing a linear gradient (250 ml each chamber) between 0.01 M ammonium bicarbonate and the same buffer 0.5 M in NaCl. Samples purified in this manner were used in amino acid composition, heme content and extinction coefficient determinations.

The Hb-L hemoglobin was dialyzed versus 1 mM sodium phosphate buffer (pH 6.6) and applied to a column (1.9 x 12 cm) of CM (carboxy methyl)-cellulose, medium mesh, (Whatman) previously equilibrated with the same buffer. The column was washed with a large volume of this buffer and the hemoglobin was eluted by developing a linear gradient (250 ml each chamber) between 1 mM sodium phosphate buffer (pH 6.6) and the same buffer 0.1M in NaCl. The Hb-L$_1$ hemoglobin fraction obtained from this treatment was used in determination of heme
content and extinction coefficient.

The Hb-H hemoglobin was concentrated by vacuum dialysis and then dialyzed versus 0.04 M sodium glycinate buffer (pH 9.5), 0.01 M in EDTA (ethylenediaminetetra-acetic acid) and applied to a column (1.9 x 90 cm) of Sephacryl S-200 equilibrated with the buffer, 0.01 M in NaCl. The column was calibrated with Blue Dextran, bovine serum albumin, ovalbumin and sperm whale myoglobin.

Isoelectric focusing of Hb-H previously dialyzed against 0.01 M Tris·HCl (pH 8.0) 0.01 M in NaCl, and 1 % in glycine and converted to the cyanmet derivative was carried out in tubes containing 5 % acrylamide gel which was 2 % in a 3:2 ratio of pH 3.5-10.5 and pH 4-6 ampholines (LKB Chem. Co.). The upper buffer reservoir contained 0.2 N NaOH and the lower buffer reservoir contained 0.01 M phosphoric acid. The gels were focused at 120 V for 12 hrs (4 °C) and then removed and scanned at 415 nm with a Zeiss PMQ II Spectrophotometer. The pH gradient was determined by slicing the gel into 0.5 cm sections and measuring the pH of each section after 24 hrs in distilled water.

Electrophoresis of globin (Teale, 1959) was performed at pH 2.2 in the presence of 6.25 M urea (Panyim and Chalkey, 1969; Poole et al., 1974). Globins were incubated overnight at room temperature in a solution
of 10 M urea (deionized with Amberlite MB-1) 5 % in acetic acid and 1 % in 2-mercaptoethanol. Pre-electrophoresis of the gels and electrophoresis of the sample were carried out at 2 mA per tube. The gels were stained with Coomassie Blue and destained with 10 % acetic acid.

Sodium dodecyl sulfate polyacrylamide electrophoresis with or without the presence of 8 M urea was carried out on 1.5 mm slab gels (Studier, 1973) with a discontinuous buffer system (Laemmli, 1970). A gel concentration of 12.5 % and a constant ratio of acrylamide to bisacrylamide of 30 : 0.8 was used. Globin or performic acid oxidized globin (Hirs, 1967) was first denatured in boiling incubation buffer (with or without 8 M urea) containing 2 % sodium dodecyl sulfate, 5 % 2-mercaptoethanol, and 1 mM PMSF (phenylmethylsulfonyl fluoride) for 1.5 min. Calibrants were ovalbumin, α-chymotrypsinogen A, sperm whale myoglobin, and lysozyme (Sigma Chem. Co.). The gels were stained in Coomassie Blue according to Fairbanks et al (1971). Globin was also denatured in boiling 6 M guanidine hydrochloride with 2-mercaptoethanol, incubated for 2 h at 37 °C and dialyzed extensively against the sodium dodecyl sulfate incubation buffer prior to analysis by sodium dodecyl sulfate electrophoresis. Apparent molecular weight of
globin denatured in 6 M guanidine hydrochloride and reduced with 5 mM dithiothreitol was determined on Sephacryl S-200 (1.4 x 83 cm) equilibrated with 6 M guanidine hydrochloride, 0.05 M Tris·HCl (pH 7.0) and 0.5 mM dithiothreitol according to Fish et al (1969). The column was calibrated with Blue Dextran, bovine serumalbumin, ovalbumin, α-chymotrypsinogen A, and sperm whale myoglobin under identical denaturing conditions.

Hemoglobin was digested with subtilisin (Carlsberg Type VIII Sigma Chem. Co.) in 0.04 M sodium glycinate buffer (pH 9.5) at 24 °C for 2 h and the reaction stopped by addition of phenylmethylsulfonyl fluoride. The concentration of enzyme used relative to hemoglobin for separation of the digestion products was 1 mg enzyme per 25 mg protein. A control aliquot of the pH 9.5 hemoglobin was treated in the same way without the addition of subtilisin. The digestion products were separated on a Sephadex G-100 column (1.9 x 114 cm) equilibrated with 0.05 M Tris·HCl (pH 8.0) buffer 0.1 M NaCl. The column was previously calibrated with Blue Dextran, bovine serum albumin, ovalbumin, α-chymotrypsinogen A and sperm whale myoglobin.

Extinction coefficients were determined by measuring the absorbance at 280 nm of a hemoglobin solution of known volume and weighing the lyophilized sample. The
heme content of hemoglobin was determined as the cyanmet-derivative according to the method of Drabkin and Austin (1935) using a 540 nm millimolar heme extinction coefficient of 11.0 for the cyanmethemoglobin (van Assendelf, 1975). The amino acid composition was carried out on purified hemoglobin according to Spackman et al (1958). Cysteine and cystine were determined as cysteic acid on performic acid oxidized globin after Hirs (1956).

Absorbance maxima were determined using a Zeiss PMQ-II spectrophotometer on freshly prepared hemoglobin. Hemoglobin was deoxygenated by addition of a small amount of sodium dithionite and carbonmonoxyhemoglobin was prepared by bubbling a hemoglobin solution with carbon monoxide gas (Matheson Co.) after the addition of a small crystal of sodium dithionite.

Oxygen binding of both the intact hemoglobins and the digested hemoglobin separated by gel chromatography were studied spectrophotometrically (Benesch et al, 1965) with a Zeiss PMQ II spectrophotometer equipped with a temperature controlled cell holder. Samples were dialyzed versus the appropriate buffer before analysis.
RESULTS

The hemoglobin of *Barbatia reeveana* is found within circulating cells in the clam's vascular system. The average blood volume is $0.35 \text{ ml} \pm 0.03 \text{ (S.D.) (N = 9)}$ per g wet weight tissue. The average hematocrit is 6.1 vol percent with a range of 2.6 to 13.1 vol percent. The hemoglobin concentration varies among clams; the hemoglobin concentration of blood pooled from 6 animals is $0.54 \text{ g per 100 ml}$. After 4 months in aquaria, most of the clams appeared anemic.

*Barbatia* hemoglobin chromatographs on Sephadex G-100 at pH 8.0 as two peaks (Fig. 1). The heavy hemoglobin fraction, Hb-H, elutes in the column void volume and accounts for approximately 40 % of the total hemoglobin eluent. The light hemoglobin fraction, Hb-L, has an apparent molecular weight of 60,000, obtained from the plot of log molecular weight versus elution volume (Fig. 2). The heavy hemoglobin fraction, Hb-H, after concentration and dialysis against either pH 7 or 8 buffer, chromatographs on Bio-gel A-5M equilibrated with the appropriate buffer as two symmetrical peaks (Fig. 3). The major Hb-H component (approximately 70 %) has an apparent molecular weight
of 430,000 and the minor Hb-H component (approximately 30%) has an apparent molecular weight of 220,000 (Fig. 4). A similar elution pattern is observed when the major 430,000 molecular weight Hb-H component is rechromatographed on the Bio-gel column, except that the 220,000 dalton Hb-H component is reduced to approximately 15% of the hemoglobin eluent.

After Bio-gel chromatography at pH 8.0 of the Hb-H hemoglobin fraction, sedimentation velocity studies were performed on both the 430,000 and 220,000 molecular weight components separately. From the plot of \( \ln r \) vs. sec, where \( r \) is the distance of the boundary from the center of rotation at time \( t \), the Hb-H 430,000 molecular weight component has a sedimentation coefficient of \( 13.6 \, S^{20,\text{w}} \), and the Hb-H 220,000 molecular component has \( S^{20,\text{w}} = 9.7 \). The Hb-H 430,000 molecular weight component also shows the presence of an additional boundary corresponding to approximately 10% of the material having a sedimentation coefficient of ca 9 S. The sedimentation velocity results obtained for the Hb-H 220,000 molecular weight component suggest that this component is homogeneous. These sedimentation results corroborate the results from gel chromatography which suggest the \textit{Barbatia} Hb-H hemoglobin contains a 430,000 molecular weight component (13.6 \( S^{20,\text{w}} \)) that tends to
dissociate into 220,000 molecular weight (9.7 $S_{20,w}$) material. There is, however, no evidence for reassociation of the 9 $S$ component into a larger structure.

Subsequent to Sephadex G-100 chromatography, isoelectric focusing of Hb-H cyanmethemoglobin indicates that the pigment appears heterogeneous. Scans of gels at 415 nm show a major peak corresponding to a $pI = 5.7$ with a shoulder near 5.8 and a minor peak with a $pI = 5.4$ (Fig. 5).

Barbatia Hb-H hemoglobin was dialyzed against 0.01 ammonium bicarbonate buffer and chromatographed on a DEAE-cellulose column equilibrated with the same buffer. The pigment eluted in the presence of a linear NaCl gradient as a single asymmetric peak (Fig. 6). The ratio of absorbances at 280 and 540 nm was nearly constant across the peak. The 280 : 540 ratio for the cyanmethemoglobin derivative from the pooled fractions under the bar in Figure 6 was the same as that found for Hb-H cyanmethemoglobin before passage through the DEAE-cellulose column. Hb-H hemoglobin purified in this way contains 1 heme per approximately 16,000 g protein based upon a millimolar extinction coefficient of 11 at 540 nm for the cyanmet-derivative and an extinction coefficient of 3.3 for a 1 mg/ml solution of Hb-H hemoglobin at 280 nm.
After the Sephadex G-100 chromatography, the Hb-L hemoglobin component was dialyzed against 1 mM sodium phosphate buffer and chromatographed on a CM-cellulose column equilibrated with the same buffer. The pigment eluted in the presence of a linear NaCl gradient as 3 symmetrical peaks of successively decreasing size (Fig. 7). Spectrophotometric scans of hemoglobin from the bars under each peak as shown in Figure 7 indicate that the major peak, Fraction Hb-L₁ contained little methemoglobin. Scans also showed that the second peak, Hb-L₂, contained more methemoglobin than Hb-L₁, and that the third peak, Hb-L₃, possessed more methemoglobin than either the first or second peaks. The ratios of absorbances at 280 nm and 540 nm for the cyanmethemoglobin derivatives of the fractions under the bars in Figure 7 were slightly reduced from that found for Hb-L cyanmethemoglobin before passage through the CM-cellulose column. Hb-L₂ hemoglobin purified in this manner contains 1 heme per 15-17,000 g protein based upon a millimolar extinction coefficient of 11 at 540 nm for the cyanmet-derivative and an extinction coefficient of 2.8 for a 1 mg/ml solution of Hb-L₁ hemoglobin at 280 nm.

Subsequent to the G-100 chromatography, isoelectric focusing of Hb-L cyanmethemoglobin shows one major band with a pI = 8.15 and a minor band with pI = 7.85, along
with a trace band with pI = 7.4 (Fig. 8).

The absorbance maxima of Barbatia Hb-H and Hb-L hemoglobins is given in Table 1.

Urea gel electrophoresis at pH 2.2 of purified Hb-L₁, Hb-L₂, and Hb-L₃ globins as shown in Figure 7 illustrate that each fraction consists of two sharp staining bands (Fig. 9a). When the Hb-L₁, Hb-L₂, and Hb-L₃ globin fractions are electrophoresed together in urea gels, two staining bands are apparent; this suggests that all three fractions may consist of the same two polypeptides.

Sodium dodecyl sulfate slab gel electrophoresis of globin or hemoglobin from the three Hb-L fractions obtained according to Figure 7 shows two staining bands corresponding to molecular weights of 15,000 and 16,700 (Fig. 10).

Tube gel electrophoresis of purified Hb-H globin in 6.25 M urea at pH 2.2 shows one sharp band (Fig. 9b). The amino acid composition of Barbatia Hb-H hemoglobin is given in Table 2.

The subunit structure of Barbatia Hb-H was examined under several conditions of denaturation. Sodium dodecyl sulfate slab gel electrophoresis was used to analyse protein treated in the following ways: 1. globin 2. performic acid oxidized globin 3. pigment which
had been incubated in 6 M guanidine hydrochloride at
100 °C in 2-mercaptoethanol and dialyzed versus the
sodium dodecyl sulfate incubation buffer 4. Protein
which had been treated with sodium dodecyl sulfate and
reducing agent in the presence of 8 M urea and electro-
phoresed in sodium dodecyl sulfate and urea. After
each treatment the protein electrophoresed as a single
band with an apparent molecular weight of 32,000
(Fig. 11 a-d). If the protein was incubated in 6 M
guanidine hydrochloride with 5 mM dithiothreitol for 2 h
at 37 °C and chromatographed on a column of Sephadryl
5-200 in equilibrium with 6 M guanidine hydrochloride
with 0.5 mM dithiothreitol, the protein elutes as a
single component with a molecular weight of about 37,000
(Fig. 12).

**Barbatia** Hb-H hemoglobin was dialyzed versus 0.04 M
sodium glycinate buffer (pH 9.5) with 0.1 M NaCl and
0.01 M EDTA and chromatographed on a Sephadryl column
equilibrated with the same buffer (Fig. 13). The major
peak eluted in the void volume along with a shoulder
peak too close to the void volume for determination
of its molecular weight.

**Barbatia** Hb-H hemoglobin was dialyzed against 0.04 M
sodium glycine buffer (pH 9.5) and digested for 2 hrs
at 24 °C with enzyme to protein ratios (w/w) of 1/5,
1/10, 1/25, 1/50, 1/100, 1/250, 1/500, and 1/1000.

The digestion products were analyzed by sodium dodecyl sulfate slab gel electrophoresis as shown in Figure 14. The major product obtained after digestion of the Hb-H hemoglobin with variable enzyme concentrations has an apparent molecular weight of 16,500. There appears to be a greater quantity of this 16,500 dalton material produced from Hb-H samples incubated with enzyme concentrations from 1/25 to 1/250 than for hemoglobin incubated with the higher or lower enzyme concentrations. The control hemoglobin incubated under the same conditions but without subtilisin shows one band with an apparent molecular weight of 32,000. The digested Hb-H hemoglobin samples also show 32,000 dalton staining bands corresponding to putative undigested hemoglobin. The quantity of this undigested hemoglobin is observed to decrease with increasing enzyme concentration. A staining band with an apparent molecular weight of 29,000 corresponding to the enzyme control is evident in some of the hemoglobin digests. Minor bands with molecular weights of 26,500, 23,500 and 22,500 are observed from the digested aliquots. The staining intensity of these minor bands appears to decrease with increasing enzyme concentrations. Trace bands with molecular weights less than the major 16,500 dalton
digested hemoglobin product are present near the dye front of the gel and may represent digested polypeptide fragments.

*Parbatia* Hb-H hemoglobin digested under the previous conditions with a 1/25 enzyme concentration produced fragments which chromatograph on Sephadex G-100 at pH 8.0 as shown in Figure 15. The major peak (Fraction I) elutes in the void volume and the apparent molecular weights of Fraction II and Fraction III are 57,000 and 30,000, respectively. When the Hb-H hemoglobin is incubated with a 1/10 enzyme concentration, the amount of 30,000 and 57,000 molecular weight materials is increased. Analyses by sodium dodecyl sulfate slab gel electrophoresis of controls and fractions under the bars as shown in Figure 15 are presented in Figure 16. Fraction I (void volume) consists of a major band with an apparent molecular weight of 32,000 along with 2 trace bands with molecular weights of 27,000, 23,000 and a minor 17,000 dalton band. Fraction II shows a major band with a molecular weight of 17,000 and a trace band of 32,000 daltons. Fraction III shows only one band corresponding to a molecular weight of 17,000.

When 2-mercaptoethanol is excluded from the sodium dodecyl sulfate incubation buffer there is no apparent change in the sodium dodecyl sulfate slab gel.
electrophoresis results from those presented in Figure 15. The ratio of absorbances at 280 nm and 415 nm for Fractions I, II and III from Figure 14 are similar to that of the intact Hb-H pigment suggesting that each fraction contains 1 heme per approximately 16,000 g protein.

*Barbatia* Hb-H hemoglobin that has been purified by Sephadex G-100 chromatography has a pH independent oxygen equilibrium curve in the range pH 7.2-8.0, with a value $P_{50} = 36 \pm 3.0$ mm Hg at 20 °C (Fig. 17a). Below pH 7.2 the oxygen affinity of Hb-H hemoglobin appears variable. The Hb-H hemoglobin exhibits moderate cooperativity that is independent of pH in the range pH 6.85 to 8.0 with a Hill coefficient of $n_H = 1.8 \pm 0.3$ at 20 °C (Fig. 17b). Spectrophotometric scans before and after oxygen equilibrium experiments showed that little methemoglobin was formed.

The effect of temperature on the oxygen binding behavior of Hb-H hemoglobin at pH 8.0 and pH 7.2 is shown by van't Hoff plots in Figure 18. At pH 8.0 the plot appears linear; the overall heat of oxygenation, including the heat of solution of oxygen, for the 15-35 °C temperature range is $\Delta H = -4.5$ kcal/mole. At pH 7.2, the plot is nearly linear from 15 to 25 °C, and the heat of oxidation is also $\Delta H = -4.5$ kcal/mole.
However, above 25 °C the oxygen equilibrium of Hb-H hemoglobin at pH 7.2 shows little temperature sensitivity: from 25 to 30 °C the value \( \Delta H = -1.6 \) kcal/mole and for the temperature range 30-35 °C the value \( \Delta H = +0.1 \) kcal/mole. The amount of cooperativity is observed to increase slightly (\( N_2 = 1.6 \) at 15 °C to \( N_2 = 2.1 \) at 35 °C) for oxygen binding studies on Hb-H carried out at pH 8.0. Temperature has no apparent effect on cooperativity of Hb-H at pH 7.2 (Fig. 19).

The oxygen equilibrium of Barbatia Hb-L that has been purified by Sephadex G-100 chromatography is pH independent in the range pH 6.8-7.6 with a \( P_{50} = 17.5 \pm 0.6 \) mm Hg at 20 °C (Fig. 20a). A slight reverse Bohr effect with \( \phi = +0.2 \) is suggested from pH 7.6 to 8.0. There is no effect of pH on the strong cooperativity of Hb-L with \( N_2 = 2.2 \pm 0.2 \) (Fig. 20b).

The effect of temperature on the oxygen equilibrium half-saturation values of Hb-L hemoglobin at pH 7.2 and pH 8.0 is shown in Figure 21. At pH 7.2 for the temperature range 15-25 °C the heat of oxygenation, including the heat of solution of oxygen, is \( \Delta H = -1.5 \) kcal/mole; for 25 to 35 °C the value \( \Delta H = -8.7 \) kcal/mole. At pH 8.0 oxygen equilibrium studies on Hb-L indicate that in the temperature interval 15-30 °C the heat of oxygenation value \( \Delta H = -7.3 \) kcal/mole. The oxygen
binding behavior of Hb-L for the temperature interval 30-35 °C at pH 8.0 has a value $\Delta H = +0.3$ kcal/mole. The amount of cooperativity of Hb-L hemoglobin at pH 7.2 appears to be independent of temperature. At pH 8.0 in the lower temperature range 15-25 °C, the cooperativity of Hb-L also appears independent of temperature, but, is observed to decrease from $N_{1/2} = 2.3$ at 25 °C to $N_{1/2} = 1.46$ at 35 °C (Fig. 22).

Oxygen equilibrium determinations were performed on the Hb-H subtilisin digested hemoglobin Fraction III isolated according to Figure 14. Fraction III has a $P_{50} = 12.8$ mm Hg with a $N_{1/2} = 1.7$. The oxygen affinity of this digested hemoglobin is much greater than the intact pigment, although the amount of cooperativity remains unchanged under the same oxygen binding conditions.
DISCUSSION

The bivalve mollusc *Barbatia reeveana* (Orbigney) has a zoogeographical range from Manuela Lagoon, Baja California to Zorritos, Peru. This species is found from the rocky intertidal to depths of 120 m (Keen, 1971). The clam is common in aggregates between rocks in the intertidal where the shell is often deformed from constriction by the substrate. *Barbatia* attaches to the substrate by a thick lamellar byssus that is secreted from glandular grooves in the foot (Kusakabe and Kitamori, 1948). In the intertidal and aquaria, I observed that *Barbatia* actively responds to changes in illumination by rapidly retracting its foot and closing the valves. Similar photosensitivity has been observed in other arcid species (Braun, 1954). Although there is little known concerning the autecology of *Barbatia*, Bretsky (1967) reported that *Barbatia domingensis* was most abundant in turbulent environments and, on the Bermuda Platform, appeared to be limited by suitable coral or rock substrate for attachment.

The erythrocytes of arcid clams are generally thin, slightly biconvex, nucleated cells, containing hemoglobin throughout the cytoplasm (Dawson, 1933).
Freadman and Mangum (1976) reported that the red cells of *Noetia ponderosa* are 17 microns in diameter and possess a single nucleus. Ochi (1979) found that the erythrocytes of *Barbatia viresceas* are 20 microns in diameter and in the electron microscope show mitochondria, endoplasmic reticulum, lysozomes, golgi, and occasionally more than two nucleii.

The mean hematocrit of *Barbatia reeveana* reported here is 6.1 vol %, which is similar to the mean value of 6.5 vol % reported for *Anadara inflata* blood (Nicol, 1966). Both *Barbatia* and *Anadara* have slightly lower blood cell volumes than *Noetia ponderosa* with a mean hematocrit of 8.11 vol % (Freadman and Mangum, 1976).

The concentration of hemoglobin in the blood of *Barbatia* was observed to vary among individuals. Some individuals exhibited severe anemia, particularly clams held in aquaria for a considerable time (4 months). Fox (1955) reported that the hemoglobin concentrations of some gastropod molluscs depends upon the ambient oxygen tension. The hemoglobin content of large *Anadara inflata* individuals was found to be greater than that of smaller animals (Kawamoto, 1928). A size versus hemoglobin content relationship was not determined for *Barbatia*.

The hemoglobin concentration of *Barbatia* blood
pooled from 6 clams is 0.54 g/100 ml. This quantity is low compared to the values of 3.49 g/100 ml given by Freadman and Mangum (1976) for *Noetia ponderosa* and 1.9 g/100 ml reported by Sato (1931) for *Anadara inflata*. The concentration of intracellular hemoglobin in the blood of *Barbatia* is also lower than that reported by Manwell (1963) for the extracellular hemoglobin concentration of *Cardita floridana* (1-2 g/100 ml) and that given by Terwilliger et al (1978a) for the extracellular hemoglobin of *Cardita borealis* (1.9 mg/ml).

*Barbatia* hemoglobin can be fractionated by Sephadex G-100 chromatography into two molecular weight components. The absorbance maxima of these two hemoglobins, Hb-L and Hb-H, are similar to the spectra of other hemoglobins (Lemberg and Legge, 1949).

The light hemoglobin fraction, Hb-L has an apparent molecular weight of 60,000. Urea gel electrophoresis of this 60,000 molecular weight pigment showed two sharp staining bands which suggests that the molecule consists of two polypeptides. Sodium dodecyl sulfate slab gel electrophoresis results showed that Hb-L is apparently constructed to two subunits with molecular weights of 15,000 and 16,700. The native 60,000 dalton pigment was found to contain 1 heme per 15-17,000 g protein. These findings suggest that *Barbatia* Hb-L is probably
a tetramer with a $\alpha_2 \beta_2$ structure. A hemoglobin with similar structure was reported by Furuta et al. (1977) for Anadara broughtonii. Tetrameric hemoglobins with $\alpha_2 \beta_2$ structures are distributed widely among vertebrates, but there are few reports of these structures found within invertebrates. The arcid bivalves appear unusual in this regard since, with the exception of Noetia ponderosa (Freadman and Mangum, 1976), they generally possess a tetramer component with two dissimilar polypeptide chains (Furuta et al., 1977; Djangmah et al., 1978).

Barbatia Hb-L was separated by cation-exchange chromatography into three heme-containing fractions. These proteins appeared identical by urea gel and sodium dodecyl sulfate slab gel electrophoresis. Absorbance measurements, however, indicated that these hemoglobins differed from each other by the quantity of methemoglobin present. This implies that Hb-L may have been separated by cation-exchange chromatography according to heme oxidation state differences among the Hb-L molecules. Isoelectric focusing of Hb-L cyanmethemoglobin, however, also suggests that the pigment is heterogeneous. These results suggest that the quaternary structure of Hb-L may exhibit conformational heterogeneity.

Perhaps the most interesting aspect of the
respiratory pigment of Barbatia is the nature of Hb-H. The heavy hemoglobin fraction, Hb-H, consists of a major 430,000 molecular weight component with a sedimentation coefficient of $S_{20,w} = 13.6$. This 430,000 molecular weight polymer seems to dissociate into 220,000 dalton heme-containing material (9.7 $S_{20,w}$). The 220,000 molecular weight structure does not appear to reaggregate into the larger molecule. This 430,000 molecular weight hemoglobin is strikingly larger than the molecular weight of any intracellular hemoglobin previously studied.

Barbatia Hb-H chromatographs on an anion-exchange column as a single asymmetrical peak suggesting microheterogeneity. Isoelectric focusing of Hb-H cyanmethemoglobin indicates that the pigment appears heterogeneous. This heterogeneity may be due to the concurrence of the 430,000 dalton polymer with its putative dissociation product.

Urea gel electrophoresis of Hb-H showed one sharp band which implies that the heavy hemoglobin component of Barbatia consists of one polypeptide. This polypeptide of Hb-H is distinguished by urea gel electrophoresis from the polypeptides constituting the tetramer (Hb-L). From these findings it seems unlikely that the occurrence of the large Hb-H hemoglobin is the
result of aggregation of the more typical Hb-L tetramer, or its subunits. Instead, as discussed below, the subunit of Hb-H is unlike that seen for any other intracellular hemoglobin studied so far.

Although Hb-H contains 1 heme per approximately 16,000 g protein, the smallest subunit produced by a number of harsh denaturing reagents has a molecular weight of 32,000 by sodium dodecyl sulfate slab gel electrophoresis. Additionally, the Hb-H subunit appeared as a 37,000 dalton fraction by Sephacryl S-200 chromatography in 6 M guanidine hydrochloride with reducing agent. These results are consistent with the hypothesis that the 32,000 dalton subunit is constructed of two covalently linked oxygen binding domains.

If the intact Hb-H pigment is digested with the protease subtilisin prior to sodium dodecyl sulfate slab gel electrophoresis, a major staining band corresponding to 16,500 daltons is observed. The molecular weight of this digestion product resembles the molecular weight of one Hb-H heme containing unit ($M_r = 16,000$). The digested hemoglobin also showed three minor bands with molecular weights between those of the intact subunit ($M_r = 32,000$) and the putative domain ($M_r = 16,500$). These minor bands may correspond to polypeptide fragments produced by digestion of the
subunit at sites other than the link connecting the
two domains. The subunit appears to be most susceptible
to digestion by subtilisin at the region linking the
two domains.

Chromatography by Sephadex G-100 of the digested
hemoglobin yielded a 30,000 dalton heme-containing
fraction that, when analyzed by sodium dodecyl sulfate
electrophoresis, was shown to consist of a 17,000
dalton polypeptide. One possible explanation for this
is that the covalent bond linking the domains was
cleaved by the enzyme, but that the denaturing agent
(sodium dodecyl sulfate) was required to dissociate
this cleaved subunit into monodomains. Gel filtration
of the digested hemoglobin also yielded a 57,000 dalton
heme-containing fraction; sodium dodecyl sulfate slab
gel electrophoresis of this fraction showed mostly
17,000 dalton with some 32,000 dalton materials. This
57,000 dalton column isolated fraction of the digested
hemoglobin probably consists of dimers of cleaved or
undigested subunits. The void volume fraction of the
chromatographed digested hemoglobin was shown by sodium
dodecyl sulfate slab gel electrophoresis to consist
mostly of the 32,000 dalton subunit. From these analyses
of the digested hemoglobin, it appears that the covalently
linked oxygen binding domains of the subunit may be
additionally held together by non-covalent bonds, possibly similar to those bonds that stabilize a typical dimeric hemoglobin.

Each 16,500 dalton oxygen binding domain of Barbatia Hb-H is similar in molecular weight to the typical myoglobin-like subunit of many invertebrate and all vertebrate hemoglobins. The didomain structure proposed for the subunit of Hb-H Barbatia hemoglobin is similar to the subunit structure reported for the extracellular hemoglobin of the branchiopod crustacean Lepidurus bilobatus (Dangott and Terwilliger, 1979). Lepidurus extracellular hemoglobin has a molecular weight of 680,000 with a 33-34,000 molecular weight subunit composed of 14,800 and 16,500 molecular weight oxygen binding domains (Dangott and Terwilliger, 1979).

The bivalve mollusc Cardita borealis contains a large \( M_r = 12 \times 10^6 \) extracellular hemoglobin that has a 300,000 dalton subunit consisting of 15-17,000 dalton oxygen binding domains (Terwilliger and Terwilliger, 1978a). The planorbid snails Helisoma and Planorbis contain large extracellular hemoglobins \( M_r = 1.7 \times 10^6 \) with subunits of 175,000 daltons (Waxman, 1975; Wood and Mosby, 1975; Terwilliger et al, 1976) composed of 15,000 molecular weight domains (Terwilliger et al, 1978). It has been suggested (Terwilliger et al, 1978)
that these large extracellular molluscan hemoglobins are composed of subunits which contain linearly linked oxygen binding domains similar to the molluscan hemocyanins (Brouwer, 1976). The structure of Barbatia Hb-H, while resembling some of these extracellular pigments, is unique among any intracellular hemoglobin previously studied. It is very large for an intracellular hemoglobin ($M_r = 430,000$), and has a subunit of 32,000 daltons consisting of two oxygen binding domains.

The oxygen equilibrium of Barbatia Hb-L shows strong cooperativity that is independent of pH with $N_2 = 2.2$ at 20 °C. The oxygen affinity of this tetramer has a $P_{50} = 17.5$ mm Hg at 20 °C that is unaffected by pH in the range pH 6.8 - 7.6. At pH 8.0 the oxygen half-saturation value is $P_{50} = 21.5$ mm Hg; this apparent reverse Bohr effect has a value $\phi = 0.2$.

Several other arcid species have tetramers that display similar cooperativities but higher oxygen affinities than those displayed by the tetramer of Barbatia. The tetramer of Anadara senilis has a $P_{50} = 8.0 - 13.0$ mm Hg at 28 °C (Djangmah et al, 1978), whereas the tetramer of Barbatia has a $P_{50} = 27.7 - 35.5$ mm Hg at 30 °C, depending on pH. Ohnoki et al (1973) reported that Anadara satowi tetrameric hemoglobin has
a $P_{50} = 11.0$ mm Hg with $N_2 = 2.1$ at $20^\circ$C; this tetramer displays a reverse Bohr effect similar to Barbatia, but at acid pH values. Although the tetramer of Anadara broughtonii exhibits no heterotropic binding properties, it shows strong cooperativity with $N_2 = 2.8 - 3.0$ (Furuta et al, 1977).

The oxygen binding properties of Barbatia Hb-L varied depending upon the experimental pH and temperature. At pH 7.2 the cooperativity of Hb-L was observed to decrease slightly with increasing temperature above $25^\circ$C. A similar decrease in cooperativity with increasing temperature was reported for hemoglobin extracts from three Anadara species by Collett and O'Gower (1972). The oxygen affinity of Barbatia Hb-L at pH 8.0 decreased with increasing temperature in the range 15 - 30 $^\circ$C ($\Delta H = -7.3$ kcal/mole). Similarly, the heat of oxygenation of Hb-L at pH 7.2 for the temperature interval 25 - 35 $^\circ$C is $\Delta H = -8.7$ kcal/mole. These thermal characteristics of Barbatia Hb-L are like those seen for vertebrate hemoglobins, that is, the oxygen affinity decreases with increasing temperature (Antonini, 1965). The heats of oxygenation for Hb-L given above are also near the range (-9 to -14 kcal/mole) reported for other hemoglobins (Rossi-Fanelli et al, 1964). However, under some experimental conditions
the oxygen equilibrium of Hb-L displayed low temperature sensitivity; at pH 7.2 from 15 - 25 °C the heat of oxygenation $\Delta H = -1.5$ kcal/mole. Freedman and Mangum (1976) suggested that the low temperature dependence of Noetia ponderosa hemoglobin at 10 °C ($\Delta H = -2.12$ kcal/mole) may permit this pigment to operate at high blood $P_{O_2}$'s, which would be expected to occur at low environmental temperatures. A possible physiological role for hemoglobin with temperature independent behavior at high environmental temperatures is discussed below.

The large molecular weight fraction of Barbatia hemoglobin (Hb-H) has a very low oxygen affinity ($P_{50} = 36$ mm Hg at 20 °C pH 7.2 - 8.0) compared to other intracellular and extracellular hemoglobins. The oxygen affinity of Hb-H is independent of pH in the range 7.2 - 8.0, but may be slightly variable below pH 7.2. The pigment displays moderate cooperativity ($N_t = 1.8$ at 20 °C) that is independent of pH at 20 °C.

Oxygen equilibrium studies of Barbatia Hb-H were also carried out at a variety of temperatures. At pH 8.0 the cooperativity of Hb-H was observed to increase slightly with increasing temperature above 25 °C. The heat of oxygenation for the pigment at pH 8.0 in the temperature range 15 - 35 °C has a value $\Delta H = -4.5$ kcal/mole. At pH 7.2 in the temperature range 15 - 25 °C
the heat of oxygenation is the same as that observed for the pigment at the higher pH. These heats of oxygenation suggest that the oxygen affinity of the pigment under the above conditions show a lower sensitivity to changes in temperature than most other hemoglobins. Weber (1972) reported that the low value of \( \Delta H = -5.3 \) kcal/mole for hemoglobin from the lugworm Arenicola marina indicates that the oxygen affinity of this pigment is relatively insensitive to changes in environmental temperature.

Remarkably, at pH 7.2 and above 25 °C, Barbatia Hb-H displays very little sensitivity to temperature: \( \Delta H = -1.6 \) kcal/mole from 25 to 30 °C and \( \Delta H = +0.1 \) kcal/mole from 30 to 35 °C. This unusually low temperature sensitivity of Barbatia Hb-H hemoglobin at high temperature was observed to occur at pH 7.2, within the range (pH 7 - 7.3) given by Freadman and Mangum (1976) for the intracellular pH of the erythrocyte of the clam Noetia ponderosa. This suggests that the relatively temperature independent oxygen binding behavior of Barbatia observed experimentally at pH 7.2 may serve as a physiological adaptation in a thermally fluctuating environment. Data concerning the oxygen content of the clam's blood at different ambient temperatures is necessary in order to lend support to this hypothesis.
However, some speculative discussion concerning this hypothesis is presented below.

Barbatia is subject to wide seasonal variations in environmental temperatures with some habitat areas in the Gulf of California reaching over 30 °C (Robinson, 1974). During these periods of high temperatures the clam would be expected to tolerate a reduction in available dissolved oxygen while its metabolic demand for oxygen concomitantly increases. The low temperature sensitivity of Barbatia Hb-H at high environmental temperatures may be important in subtidal habitats where low levels of available oxygen are likely to occur during summer. Collett and O'Gower (1972) found that hemoglobin extracts from three Anadara bivalve species show an increase in $P_{50}$ values with increasing temperature from 15 to 20 °C, but that in the range 20 to 30 °C, the oxygen affinity varied inversely with temperature. Collett and O'Gower (1972) proposed that this unusual thermal characteristic of Anadara hemoglobin is an adaptation to increase the oxygen gradient across the ctenidia when the clam is in warm oxygen depleted water.

In Barbatia, the low temperature sensitivity of Hb-H hemoglobin may facilitate the complete oxygenation of this low affinity pigment, especially when the
clam is subjected to high ambient temperatures. The striking temperature insensitivity of Barbatia hemoglobin may function to facilitate the uptake of oxygen by the clam and be responsible, in part, for the animal's ability to be eurythermal.

The results of oxygen equilibrium studies show that the isolated 30,000 dalton subtilisin-digested hemoglobin fraction combines reversibly with oxygen. The affinity and cooperativity of this digestion product appear consistent with its proposed structure; that is, the 30,000 dalton putative cleaved but undissociated didomain displays oxygen binding properties that appear to resemble those of a typical dimeric clam hemoglobin (Anadara satowi Hb I has a $P_{50} = 10$ mm Hg and $N = 1.5$) (Ohnoki et al, 1973). The oxygen affinity of the cleaved but undissociated didomain is greater than that of the undigested pigment while the amount of cooperativity is the same. Although the oxygen binding properties of the uncleaved didomain are not known, the intact subunit may be expected to display a relatively high affinity similar to that of the cleaved but undissociated didomain. The oxygen binding properties of the monodomain are not known.

Oxygen equilibrium studies on Helisoma subtilisin-generated 17-19,000 dalton domains and linked domains
show, in contrast to the intact pigment, high oxygen affinity and no cooperativity or Bohr effect (Terwilliger et al, 1977). On the other hand, isolated domains of Cardita borealis extracellular hemoglobin show oxygen binding properties that are similar to the intact clam pigment (Terwilliger and Terwilliger, 1978a). Although the homotropic and heterotropic oxygen binding characteristics of the intact didomain of Barbatia are not known, the function of the pigment seems to depend upon its intact quaternary structure. In this regard Barbatia Hb-H hemoglobin behaves more like Helisoma hemoglobin than Cardita hemoglobin.

The erythrocytes of Barbatia contain two hemoglobins that have different molecular weights and oxygen affinities. The lighter fraction, Hb-L, has a $P_{50} = 17.5$ mm Hg at 20 °C in the pH range 6.9 - 7.6 and, the heavier fraction, Hb-H, has a $P_{50} = 36$ mm Hg at 20 °C in the range pH 7.2 - 8.0. The co-occurrence of these two hemoglobins with considerably different oxygen affinities suggest that the concomitant presence of the two hemoglobins in the clam may allow the transport of oxygen over a wide range of environmental oxygen tensions. Since Barbatia is abundant in the wave exposed rocky intertidal habitat where surface waters are probably well oxygenated, the low oxygen affinity of Hb-H seems
adaptive, and suggests that the pigment functions in oxygen transport rather than storage. The Hb-L hemoglobin component, with a higher oxygen affinity, may serve a role in oxygen transport or storage during periods of low tide. Alternately, Barbatia may utilize pathways of anaerobic metabolism during periods when it is unable to ventilate, as has been reported for other bivalves (de Zwaan and Wijsman, 1975). Further physiological studies are required before an actual physiological function can be prescribed for the hemoglobins of Barbatia.

Several of the arcid bivalves possess intracellular tetrameric and dimeric hemoglobins (Furuta et al, 1977). Barbatia contains a tetramer but lacks the typical arcid dimer. Instead, Barbatia erythrocytes contain a unique large molecular weight hemoglobin constructed of a didomain subunit. One is tempted to speculate that this subunit may be evolutionarily related to the dimer of other arcids derived, perhaps, from the tandem duplication of a globin genome as has been suggested for other large domain structures (Terwilliger and Terwilliger, 1978a). In this respect, sequence studies comparing the domain of Barbatia and the polypeptide of a typical arcid dimer would be interesting. The large molecular weight intracellular hemoglobin found
in the red cells of *Barbatia* is unique, and appears to be an excellent pigment for further study of domain structure and synthesis.
Figure 1. Chromatography of *Barbatia roeveana* hemoglobin on Sephadex G-100. Buffer 0.05 M Tris·HCl (pH 8.0) 0.01 M in NaCl. \( \bullet \) = absorbance 540 nm. Protein calibration markers: (a) Blue Dextran, (b) bovine serum albumin, (c) ovalbumin, (d) \( \alpha \)-chymotrypsinogen A, (e) sperm whale myoglobin.
Figure 2. Molecular weight of *Barbatia reeveana* hemoglobin on Sephadex G-100. Data is from Figure 1. Protein calibration markers are (a) Blue Dextran, (b) bovine serum albumin, (c) ovalbumin, (d) α-chymotrypsinogen A, (e) sperm whale myoglobin. The arrows indicate the volume at which the hemoglobins elute from the column.
Figure 3. Chromatography of *Barbatia reeveana* Hb-H hemoglobin fraction on Bio-gel A-5M. ○ = Absorbance at 415 nm. Buffer: 0.05 M Tris·HCl (pH 7.0) 0.01 M NaCl, 0.01 M MgCl$_2$. Protein calibration markers are (b) *Cancer magister* hemocyanin, (c) *Cancer magister* hemocyanin, (d) bovine heart lactate dehydrogenase.
FIGURE 3

ELUTION VOLUME (mL)

VOLUME ELUTION

b

140

160

c

180

d

ABSORBANCE
Figure 4. Molecular weight of *Barbatia reeveana* Hb-H hemoglobin fraction on Bio-gel A-5M. Data is from Figure 3. Arrows indicate the volume at which the hemoglobins elute from the column. Calibration proteins are (a) *Eudistylia vancouveri* chlorocruorin, (b) *Cancer magister* hemocyanin, (c) *Cancer magister* hemocyanin, (d) bovine heart lactate dehydrogenase.
Figure 5. Spectrophotometric scan of isoelectric focused Barbatia reeveana Hb-H cyanmethemoglobin at 415 nm. Gel concentration 5% acrylamide.
FIGURE 5
Figure 6. DEAE-cellulose chromatography of *Barbatia roeveana* Hb-H hemoglobin. Δ, Absorbance at 540 nm, ○, Absorbance at 280 nm. Buffer: 0.01 M NH₄HCO₃. The pigment elutes in the presence of a 0-0.5 M NaCl linear gradient.
Figure 7. CM-cellulose chromatography of *Barbatia reeveana* Hb-L hemoglobin. △, Absorbance 540 nm, ○, Absorbance 280 nm. Buffer: 1 mM sodium phosphate (pH 6.6). The pigment elutes in the presence of a 0-0.1 M NaCl linear gradient.
Figure 8. Spectrophotometric scan of isoelectric focused *Barbatia reeveana* Hb-L cyanmethemoglobin. Gel concentration 5% acrylamide.
Figure 9. Urea gel electrophoresis patterns of Barbatia reeveana hemoglobins. A. and B.: 
A. Barbatia Hb-L hemoglobin fraction; B. Barbatia Hb-H hemoglobin fraction.
Figure 10. Sodium dodecyl sulfate slab gel electrophoresis of *Barbatia reeveana* Hb-L hemoglobin fraction. Gel concentration 12.5 %.
FIGURE 10

43,000
25,700
17,200
14,300
Figure 11. Sodium dodecyl sulfate slab gel electrophoresis of *Barbatia reeveana* Hb-H hemoglobin fraction.

A-D:  
A. *Barbatia* Hb-H globin;  
B. *Barbatia* Hb-H performic acid oxidized globin;  
C. *Barbatia* Hb-H globin previously denatured in 6 M guanidine hydrochloride;  
D. sodium dodecyl sulfate slab gel electrophoresis in 8 M urea of *Barbatia* Hb-H globin.

Gel concentration 5 %.
Figure 12. Chromatography of *Barbatia reeveana* Hb-H hemoglobin fraction on Sephacryl S-200. Absorbance at 280 nm. Buffer 0.05 M Tris·HCl (pH 7.0) 6 M guanidine hydrochloride, 0.5 mM dithiothreitol. Hb-H globin previously reduced. Calibration markers are (a) bovine serum albumin, (b) ovalbumin, (c) α-chymotrypsinogen A, (d) sperm whale myoglobin, (e) potassium ferricyanide.
FIGURE 12

ELUTION VOLUME (ml)

ABSORBANCE

a b c d e

40 80 120
Figure 13. Chromatography of *Barbatia reeveana* Hb-H hemoglobin on Sephacryl S-200. •, Absorbance at 540 nm. Buffer: 0.04 M sodium glycinate (pH 9.5), 0.1 M NaCl, 0.01 M EDTA. Calibrant markers: (a) Blue Dextran, (b) bovine serum albumin, (c) ovalbumin, (d) sperm whale myoglobin.
Figure 15. Chromatography on Sephadex G-100 of 2 h subtilisin digested Barbatia reeveana Hb-H hemoglobin (1 mg enzyme to 25 mg protein, 24 °C). Δ, Absorbance at 415 nm. ○, Absorbance at 280 nm. Buffer; 0.05 M Tris·HCl (pH 8.0) 0.1 M NaCl. Calibration markers: (a) Blue Dextran, (b) bovine serum albumin, (c) ovalbumin, (d) α-chymotrypsinogen A, (e) sperm whale myoglobin.
Figure 16. Sodium dodecyl sulfate slab gel electrophoresis of products isolated from subtilisin digested *Barbatia reeveana* Hb-H hemoglobin. Gel concentration 12.5%. A. hemoglobin control, B. whole digest aliquot, C. Fraction I, D. Fraction II, E. Fraction III. The fractions are from hemoglobin obtained as in Figure 15.
Figure 17. Effect of pH on the oxygen equilibrium of *Barbatia reeveana* Hb-H hemoglobin at 20 °C: A and B.

A. Effect of pH on *Barbatia reeveana* oxygen affinity,
B. Effect of pH on *Barbatia reeveana* cooperativity.
Figure 18. Effect of temperature on the oxygen affinity of *Barbatia reeveana* Hb-H hemoglobin at pH 7.2 and pH 8.0. O, pH 7.2. □, pH 8.0. Temperatures: 15 to 35 °C. Buffer: 0.05 M Tris·HCl, 0.1 M NaCl. Values represent the mean of two experiments.
Figure 19. Effect of temperature on the cooperativity of *Barbatia reeveana* Hb-H hemoglobin at pH 7.2 and pH 8.0. O, pH 7.2. Δ, pH 8.0. Buffer: 0.05 M Tris HCl, 0.1 M NaCl. Temperatures 15-35°C.
FIGURE 19

![Graph showing data points and temperature scale. The x-axis represents TEMP °C ranging from 15 to 35, and the y-axis represents N \sqrt{2} ranging from 1.0 to 2.5. Different symbols represent different data sets.](image-url)
Figure 20. Effect of pH on the oxygen equilibrium of *Barbatia reeveana* Hb-L hemoglobin at 20°C: A and B.

A. Effect of pH on the oxygen affinity of Hb-L;

B. Effect of pH on the cooperativity of Hb-L.
Figure 21. Effect of temperature on the oxygen affinity of *Barbatia reeveana* Hb-L hemoglobin at pH 7.2 and pH 8.0; O, pH 7.2. □, pH 8.0. Buffer: 0.05 M Tris-HCl, 0.01 M NaCl. Temperatures 15-35 °C. Values represent the mean of two experiments.
Figure 22. Effect of temperature on the cooperativity of *Barbatia reeveana* Hb-L hemoglobin at pH 7.2 and pH 8.0. ○, pH 7.2; △, pH 8.0. Buffer: 0.05 M Tris·HCl, 0.1 M NaCl. Temperatures 15-35 °C.
FIGURE 22

TEMP °C

N \sqrt{2}

15 20 25 30 35
TABLE 1. ABSORBANCE MAXIMA OF *BARBATIA REEVEANA* Hb-H AND Hb-L HEMOGLOBINS

<table>
<thead>
<tr>
<th></th>
<th>max</th>
<th>max</th>
<th>Soret max</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb-H</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxy</td>
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<td>540</td>
<td>415</td>
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<tr>
<td>Deoxy*</td>
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<td>CO</td>
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<tr>
<td>Cyanmet</td>
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<td>540</td>
<td>421</td>
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<tr>
<td>Hb-L</td>
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<tr>
<td>Oxy</td>
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<tr>
<td>Cyanmet</td>
<td></td>
<td>540</td>
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</table>

*Deoxygenated with sodium dithionite
**TABLE 2: AMINO ACID COMPOSITION OF BARBATIA REEVEANA**

Hb-H HEMOGLOBIN

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mol %</th>
<th>Amino acid</th>
<th>Mol %</th>
</tr>
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<tbody>
<tr>
<td>Lys</td>
<td>10.4</td>
<td>Ala</td>
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<tr>
<td>His</td>
<td>1.7</td>
<td>Half cys*</td>
<td>1.8</td>
</tr>
<tr>
<td>Arg</td>
<td>6.4</td>
<td>Val</td>
<td>8.0</td>
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<tr>
<td>Asp</td>
<td>16.0</td>
<td>Met</td>
<td>1.9</td>
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<tr>
<td>Thr</td>
<td>3.2</td>
<td>Ile</td>
<td>6.0</td>
</tr>
<tr>
<td>Ser</td>
<td>3.6</td>
<td>Leu</td>
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</tr>
<tr>
<td>Glu</td>
<td>9.0</td>
<td>Tyr</td>
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<td>Pro</td>
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<td>Phe</td>
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<tr>
<td>Gly</td>
<td>6.5</td>
<td>Trp</td>
<td>**</td>
</tr>
</tbody>
</table>

* Determined as cysteic acid

** Not measured
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