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The Quaternary Structure of an Unusual High-Molecular-Weight Intracellular Haemoglobin from the Bivalve Mollusc *Barbatia reeveana*

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The arcid clam *Barbatia reeveana* contains an intracellular haemoglobin with an unusual structure. First, compared with other intracellular haemoglobins, it is extremely large, with a mol.wt. of 430000 and an $s_{20,w}$ of 13.6S. A minor component (mol.wt. = 220000; $s_{20,w}$ = 9.7S) is also present as a probable dissociation product of the major component. Secondly, this haemoglobin has an unusual subunit structure. It contains 1 mol of haem per 16000g of protein, in common with most other haemoglobins. However, the smallest polypeptide that could be obtained after treatment with sodium dodecyl sulphate or 6M-guanidine with reducing agent has a mol.wt. of 32000–37000. Digestion of the haemoglobin with the proteinase subtilisin produces both 57000- and 30000-mol.wt. aggregates that contain 1 mol of haem per 16000g of protein and that can be dissociated into 16500-mol.wt. polypeptides by treatment with sodium dodecyl sulphate. The intact polymer shows slight co-operativity ($h = 1.7$), lacks a Bohr effect between pH 7 and 8, and has a low oxygen affinity [$P_{50} = 4.8$kPa (36mmHg) at 20°C] relative to other haemoglobins. The 30000-mol.wt. aggregate obtained by digestion of the polymer binds oxygen reversibly with an affinity greater than that of the polymer, but with some co-operativity ($h = 1.7$). These results are consistent with the hypothesis that the subunits of this unusually large intracellular haemoglobin are 32000-mol.wt. polypeptides that in turn are composed of two covalently linked haem-containing oxygen-binding domains. This is the first report of an intracellular haemoglobin with such a structure.

Most intracellular haemoglobins of both vertebrates and invertebrates consist of haem-containing polypeptides with mol.wts. of 15000–17000 or dimeric and tetrameric aggregates of these polypeptides. Only a few examples of intracellular haemoglobins have been reported that are aggregates larger than tetramers; haemoglobins of some species of fish, amphibians and birds exist as octamers (Elli *et al.*, 1970; Araki *et al.*, 1974; Morrow *et al.*, 1974; Riggs, 1979), a sea-cucumber haemoglobin polymerizes in the deoxy state to octamers or larger polymers (Bonaventura & Kitto, 1972), an octameric haemoglobin has been described in the erythrocytes of the blood worm (*Glycera dibranchiata*) (Harrington *et al.*, 1978), and deoxy-(human sickle-cell haemoglobin) forms large fibrous aggregates. However, high-molecular-weight haemoglobins usually do not occur in erythrocytes. Furthermore, when they do, they all share a similar subunit mol.wt. of 15000–17000.

In the present work we have examined some physical and chemical properties of the haemoglobin from the erythrocytes of an arcid clam, *Barbatia reeveana*. This haemoglobin has some very unusual properties: not only is it extremely large for an intracellular haemoglobin, with a mol.wt. of 430000, but also it is composed of a subunit with a mol.wt. of 32000 that in turn consists of two covalently linked haem-containing oxygen-binding domains. The quaternary structure and oxygen-equilibrium properties of this unusual haemoglobin are here presented.

Experimental

Animals and preparation of haemoglobin

Barbatia reeveana (Orbigney) was collected from the wave-exposed rocky intertidal shore near El Coyote in Conception Bay, Baja California, Mexico, and identified as described by Keen (1971). The animals were transported to the laboratory, where they were kept in aerated aquaria at room tempera-

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ture (19–23°C) with daily changes of seawater (32 parts per thousand).

Blood, collected from the pallial sinus of one to five animals, was pooled and washed three times with ice-cold 3% (w/v) NaCl. The final cell pellet was resuspended in 1mM-Tris/HCl, pH8.0, and placed on ice for 1h. This solution was then centrifuged at 12000*g* for 10min. The supernatant was chromatographed on a column (1.9cm × 100cm) of Sephadex G-100 equilibrated with 0.1M-Tris/HCl buffer, pH8.0, 0.1M in NaCl. The chromatography allowed separation of the polymeric haemoglobin, which will be exclusively discussed in the present paper, from another tetrameric haemoglobin that also occurs in *B. reeveana* (N. P. Grinich & R. C. Terwilliger, unpublished work).

Molecular-weight analyses

The haemoglobin was concentrated by vacuum dialysis and dialysed against either 0.1M-Tris/HCl, pH8.0, 0.1M in NaCl, or 0.03M-Tris/HCl, pH7.0, 0.1M in NaCl and 0.01M in $MgCl_2$. The haemoglobin was applied to a column (1.9cm × 100cm) of Bio-Gel A-5M (200–400 mesh; Bio-Rad Laboratories), previously equilibrated with either of the above buffers. The Bio-Gel column was calibrated with crab (*Cancer magister*) haemocyanin (mol.wt. 9.0×10^5 and 4.5×10^5) (Ellerton *et al.*, 1970), and bovine heart lactate dehydrogenase (mol.wt. 150000) (Sigma).

Sedimentation-velocity experiments were carried out on haemoglobin in (0.1M-Tris/HCl) pH8.0/0.1M-NaCl, with a Beckman–Spinco model E ultracentrifuge equipped with a RTIC temperature-control unit and scanning optics. The temperature was 20°C and the rotor speeds were 36000rev./min for the 13.6S material (1.5mg/ml) and 44000rev./min for the 9.7S material (0.7mg/ml). Sedimentation coefficients were calculated by the method of least squares from the plot of $\ln r$ against s and corrected to the viscosity and density of water under standard conditions (Svedberg & Pederson, 1940). The apparent mol.wt. of globin denatured in 6M-guanidine hydrochloride and reduced with 5mM-dithiothreitol was determined on a column (1.4cm × 83cm) of Sephacryl S-200 equilibrated with 6M-guanidine hydrochloride, 0.03M-Tris/HCl, pH7.0, and 0.5mM-dithiothreitol as described by Fish *et al.* (1969). The column was calibrated with Blue Dextran, bovine serum albumin, ovalbumin, α-chymotrypsinogen A and sperm-whale myoglobin under identical denaturing conditions.

Electrophoresis

Isoelectric focusing of cyanmethaemoglobin previously dialysed against 0.01M-Tris/HCl, pH8.0, 0.01M in NaCl and 1% (w/v) in glycine, was carried out in tubes containing 5% (w/v) polyacrylamide gel that was 2% (v/v) in a 3:2 ratio of pH3.5–10.5 and pH4–6 Ampholines (LKB). The upper buffer reservoir contained 0.02M-NaOH and the lower buffer reservoir contained 0.01M-phosphoric acid. The gels were focused at 120V for 12h (4°C) and then removed and scanned at 415nm with a Zeiss PMQ II spectrophotometer.

Electrophoresis of globin (Teale, 1959) was performed at pH2.2 in the presence of 6.25M-urea (Panyim & Chalkey, 1969; Poole *et al.*, 1974). Globin was incubated overnight at room temperature in a solution of 10M-urea (deionized with Amberlite MB-1 resin), 5% (v/v) in acetic acid and 1% (v/v) in 2-mercaptoethanol. Pre-electrophoresis of the gels and electrophoresis of the sample were carried out at 2mA per tube. The gels were stained with Coomassie Blue and destained with 10% (v/v) acetic acid.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with or without the presence of 8M-urea was carried out on 1.5mm-thick slab gels (Studier, 1973) with a discontinuous buffer system (Laemmli, 1970). A gel concentration of 12.5% (w/v) and a constant ratio of acrylamide to bisacrylamide of 75:2 was used. Globin or performic acid-oxidized globin (Hirs, 1967) was first denatured in boiling incubation buffer (with or without 8M-urea) containing 2% (w/v) sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, and 1mM-phenylmethanesulphonyl fluoride for 1.5min. Calibrants were ovalbumin, α-chymotrypsinogen A, sperm-whale myoglobin and lysozyme (Sigma). The gels were stained in Coomassie Blue as described by Fairbanks *et al.* (1971). Globin was also denatured in boiling 6M-guanidine hydrochloride with 1% (v/v) 2-mercaptoethanol, incubated for 2h at 37°C and dialysed extensively against the sodium dodecyl sulphate/incubation buffer before analysis by electrophoresis.

Digestion with subtilisin

Haemoglobin was digested with subtilisin (Carlsberg Type VIII; Sigma) in 0.04M-sodium glycinate buffer, pH9.5, at 24°C for 2h (enzyme/protein ratio 1:25, w/w) and the reaction stopped by addition of phenylmethanesulphonyl fluoride. The digestion products were separated on a column (1.9cm × 114cm) of Sephadex G-100 equilibrated with 0.1M-Tris/HCl buffer, pH8.0, 0.1M in NaCl. The column had been previously calibrated with Blue Dextran, bovine serum albumin. ovalbumin, α-chymotrypsinogen A and sperm-whale myoglobin.

Analytical methods

The haemoglobin was dialysed against 0.01M-NH_4HCO_3 and applied to a column (1.9cm × 12cm)

of DEAE-cellulose (Sigma) equilibrated with the same buffer. The column was washed with this buffer, and the haemoglobin eluted by establishing a linear gradient (250ml in each chamber) between 0.01M-NH_4HCO_3 and the same buffer, 0.5M in NaCl. Samples purified in this manner were used in amino acid-composition, haem-content and absorption-coefficient determinations.

Absorption coefficients were determined by measuring the A_{280} of a haemoglobin solution of known volume and weighing the freeze-dried sample. The haem (as the cyanmet derivative) content was determined as described by Drabkin & Austin (1935) by using a ε_{540}^{mM} of 11.0 for cyanmethaemoglobin (van Assendelft & Ziglstra, 1975). The amino acid composition was determined on purified haemoglobin as described by Spackman *et al.* (1958). Cysteine and cystine were determined as cysteic acid on performic acid-oxidized globin as described by Hirs (1967).

Oxygen-equilibrium studies

Oxygen binding by both the intact haemoglobins and the digested haemoglobin 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 dialysed against the appropriate buffer before analysis.

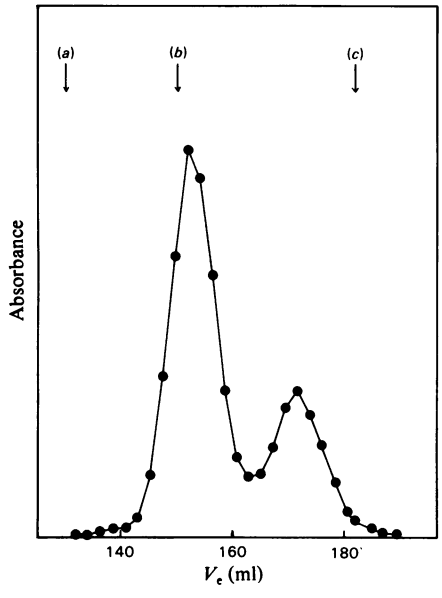

Fig. 1. *Chromatography of Barbatia polymeric haemoglobin on Bio-Gel A-5M*

●, A_{415}. V_e is the elution volume. The buffer was 0.03M-Tris/HCl, pH7.0, 0.1M in NaCl, 0.01M in $MgCl_2$. Calibration markers were: (*a*) *Cancer magister* haemocyanin (mol.wt. 940000); (*b*) *C. magister* haemocyanin (mol.wt. 450000); (*c*) bovine heart lactate dehydrogenase (mol.wt. 140000).

Results

The haemoglobin of *Barbatia reeveana* is found within circulating cells in the clam's vascular system. The pigment can be separated into two haemoglobin fractions on Sephadex G-100 at pH8.0. The smaller haemoglobin is a 60000-mol.wt. tetramer (N. P. Grinich & R. C. Terwilliger, unpublished work). Characteristics of the high-molecular-weight haemoglobin, which eluted in the void volume of the G-100 column, are presented below.

The high-molecular-weight haemoglobin chromatographs on Bio-Gel A-5M at pH7.0 or 8.0 as two peaks (Fig. 1). The major peak (about 70% of the total haemoglobin) has an apparent mol.wt. of 430000 and the minor component a mol.wt. of 220000. If the 430000-mol.wt. component is rechromatographed on the same column, 220000-mol.wt. material is again observed. Sedimentation studies showed that the 430000-mol.wt. material is heterogeneous, containing two components with $s_{20,w}$ values of 13.6S and 10.1S. The isolated 220000-mol.wt. component appears homogeneous, with an $s_{20,w}$ of 9.7S.

Isoelectric focusing of the polymer as cyanmethaemoglobin shows that it is heterogeneous. Spectrophotometric scans of gels at 415nm resolve a major component corresponding to pI5.7 with a shoulder near pI5.8 and a minor peak at pI5.4 (Fig. 2).

Barbatia haemoglobin is eluted as a single peak from a DEAE-cellulose column. The A_{280}/A_{540} ratios were constant across the peak and similar to that seen for the haemoglobin before fractionation. Haemoglobin purified in this way contains 1mol of haem per 16000g of protein. This value is based on ε_{540}^{1mM} of 11 for the cyanmet derivative and $\varepsilon_{280}^{1mg/ml}$ of 3.3 for the haemoglobin (path length 1cm).

The subunit structure of the haemoglobin was examined under several conditions of denaturation. Sodium dodecyl sulphate/slab-gel electrophoresis was used to analyse protein treated in the following ways: (1) globin (Teale, 1959); (2) performic acid oxidation; (3) incubation in 6M-guanidine hydrochloride/2-mercaptoethanol at 100°C and dialysis against sodium dodecyl sulphate and reducing agent; (4) incubation in 8M-urea followed by electrophoresis in sodium dodecyl sulphate and urea. After each of these treatments the protein electrophoreses as a single band with an apparent mol.wt. of 32000 (Figs. 3*b*–3*e*). Tube-gel electrophoresis of purified globin in 6.25M-urea, pH2.2, with 1% 2-mercaptoethanol shows a single staining band (Fig. 3*f*). When globin was incubated in 6M-guanidine hydro-

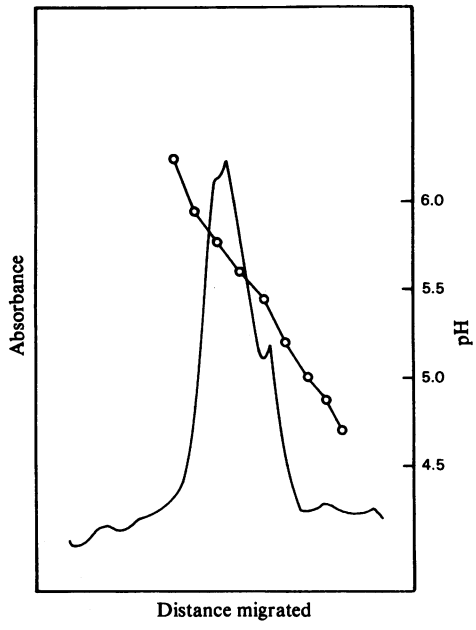

Fig. 2. *Spectrophotometric scan at 415 nm of isoelectrically focused Barbatia polymeric cyanmethemoglobin*
The gel was 5% (w/v) polyacrylamide. ——, A_{415}; O, pH values.

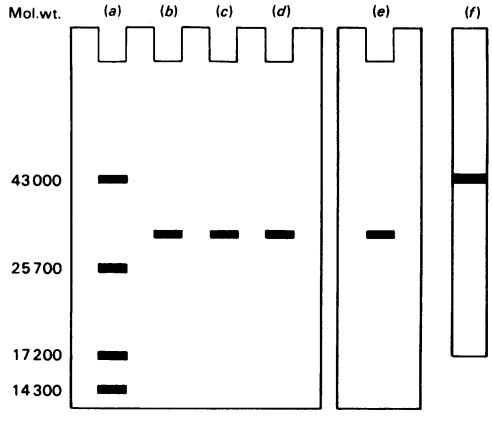

Fig. 3. *Electrophoreses of Barbatia polymeric haemoglobin*
(*a–e*) Sodium dodecyl sulphate/slab-gel electrophoresis of: (*a*) calibration markers; (*b*) *Barbatia* globin; (*c*) *Barbatia* performic acid-oxidized globin; (*d*) *Barbatia* globin previously denatured in 6 M-guanidine hydrochloride; (*e*) sodium dodecyl sulphate/slab-gel electrophoresis in 8 M-urea of *Barbatia* globin; (*f*) tube-gel electrophoresis in 6.25 M-urea of *Barbatia* globin.

chloride/5 mM-dithiothreitol for 2 h at 37°C and then chromatographed on a column in equilibrium with 6 M-guanidine hydrochloride/0.5 mM-dithiothreitol, pH 7.0, it was eluted as a major component with an apparent mol.wt. of about 37000 (Fig. 4).

The amino acid composition of DEAE-cellulose-purified haemoglobin is shown in Table 1.

Sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of oxyhaemoglobin digested with different concentrations of subtilisin is shown in Fig. 5. The major product obtained at all enzyme/protein ratios has an apparent mol.wt. of 16500. There appears to be a greater quantity of 16500-mol.wt. material produced in samples incubated with enzyme/protein ratios from 1:25 to 1:250 than with higher or lower enzyme/protein ratios. Control haemoglobin incubated under the same conditions but without subtilisin shows a single 32000-mol.wt. band (Fig. 5*b*). The digested haemoglobin samples also show some bands corresponding to 32000-

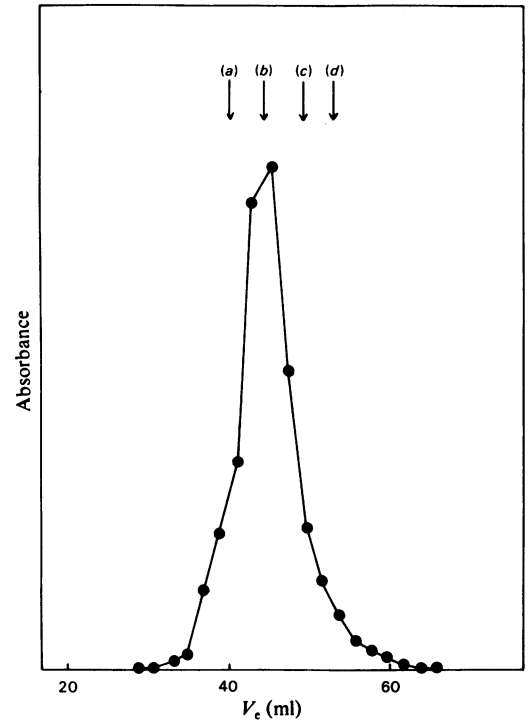

Fig. 4. *Chromatography of reduced Barbatia polymeric globin on Sephacryl S-200*
Absorbance was measured at 280 nm. V_e is the elution volume. The buffer was 0.03 M-Tris/HCl (pH 7.0)/6 M-guanidine hydrochloride/0.5 mM-dithiothreitol. Calibration markers were: (*a*) bovine serum albumin; (*b*) ovalbumin; (*c*) α-chymotrypsinogen A; (*d*) sperm-whale myoglobin.

Table 1. *Amino acid composition of Barbatia reeveana polymeric haemoglobin*

Amino acid	Residues/ 100 residues	Amino acid	Residues/ 100 residues
Lys	10.4	Ala	7.0
His	1.7	Cys*	1.8
Arg	6.4	Val†	8.0
Asp	16.0	Met	1.9
Thr‡	3.2	Ile†	6.0
Ser‡	3.6	Leu	11.0
Glu	9.0	Tyr	0.8
Pro	3.2	Phe	4.2
Gly	6.5	Trp	§

* Determined as cysteic acid.
† Corrections made for incomplete hydrolysis.
‡ Corrections made for destruction during hydrolysis.
§ Not measured.

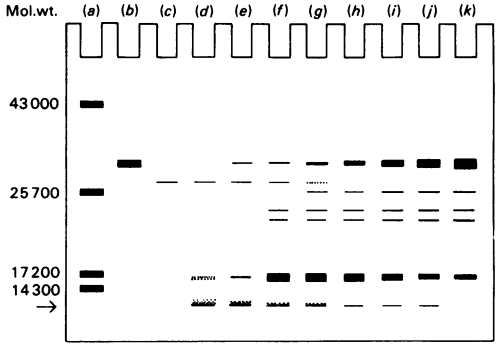

Fig. 5. *Sodium dodecyl sulphate/slab-gel electrophoresis of Barbatia polymeric haemoglobin digested with subtilisin*
(*a*) Calibration markers; (*b*) control haemoglobin; (*c*) enzyme control; (*d–k*) enzyme/protein ratios of: (*d*) 1:5; (*e*) 1:10; (*f*) 1:25; (*g*) 1:50; (*h*) 1:100; (*i*) 1:250; (*j*) 1:500; (*k*) 1:1000. The gel was 12.5% (w/v) polyacrylamide. The arrow indicates position of the dye front.

mol.wt. putative undigested haemoglobin. The amount of undigested haemoglobin decreases with increasing enzyme concentration. A staining band corresponding to an apparent mol.wt. of 29000 and to subtilisin (Fig. 5*c*) is present in the haemoglobin digested with high enzyme/protein ratios. Minor bands with mol.wts. of 26500, 23500 and 22500 are also evident. The staining intensity of these minor bands is lower in digests incubated with high enzyme concentrations. Trace bands with mol.wts. less than the major 16500-mol.wt. product are present near the dye front in the gel.

Barbatia haemoglobin digested under the above-mentioned conditions with an enzyme/protein ratio

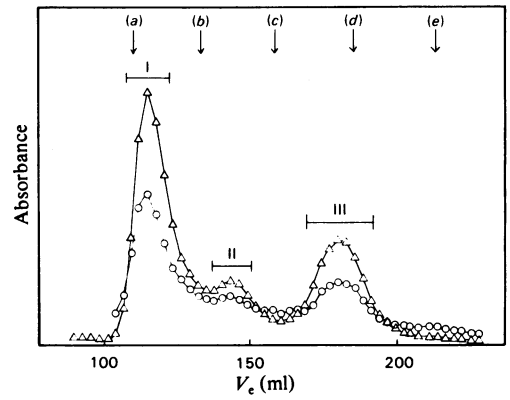

Fig. 6. *Chromatography on Sephadex G-100 of 2h-subtilisin-digested Barbatia polymeric haemoglobin (1 mg of enzyme to 250 mg of protein, at 24°C)*
△, A_{415}; ○, A_{280}. V_e is the elution volume. The buffer was 0.1 M-Tris/HCl (pH 8.0)/0.1 M-NaCl. Calibration markers were: (*a*) Blue Dextran; (*b*) bovine serum albumin; (*c*) ovalbumin; (*d*) α-chymotrypsinogen A; (*e*) sperm-whale myoglobin.

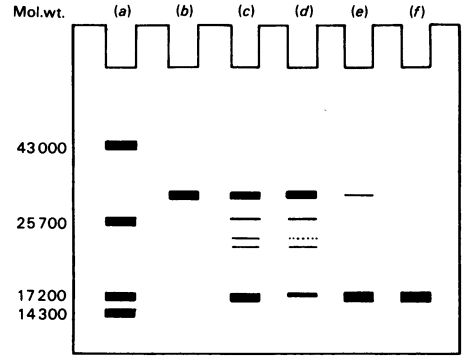

Fig. 7. *Sodium dodecyl sulphate/slab-gel electrophoresis of products isolated from subtilisin-digested Barbatia polymeric haemoglobin*
(*a*) Calibration markers; (*b*) haemoglobin control; (*c*) whole digest; (*d*) Fraction I; (*e*) Fraction II; (*f*) Fraction III. The fractions are from haemoglobin obtained as described in Fig. 7. The gel was 12.5% (w/v) polyacrylamide.

of 1:250 was chromatographed on Sephadex G-100 at pH 8.0 as shown in Fig. 6. The major peak (Fraction I) eluted in the void volume; the apparent molecular weights of Fractions II and III are 57000 and 30000 respectively. Analyses by sodium dodecyl sulphate/slab-gel electrophoresis of fractions under the bars shown in Fig. 6 are presented in Fig. 7. Undigested control haemoglobin is shown in Fig. 7(*b*). Fraction I shows a major band with an

apparent mol.wt. of 32000, two trace bands with mol.wts. of 26500 and 23500 and a small amount of 16500-mol.wt. material (Fig. 7*d*). Fraction II shows a major band with a mol.wt. of 16500 and a trace band of 32000 mol.wt. (Fig. 7*e*). Fraction III shows only one band, corresponding to a mol.wt. of 16500 (Fig. 7*f*). If 2-mercaptoethanol is omitted from the sodium dodecyl sulphate-containing incubation buffer, the results are the same as shown in Fig. 7. The A_{280}/A_{415} ratios for Fractions I, II and III from Fig. 6 are similar to that of the intact haemoglobin suggesting that each fraction contains 1 mol haem per approx. 16000 g of protein.

Oxygen-equilibrium studies of the polymeric haemoglobin purified by Sephadex G-100 show that this pigment has no Bohr effect between pH 7.2 and 8.0, with a $P_{50} = 4.8 \pm 0.4$ (S.D.) kPa[36 ± 3 (S.D.) mmHg] at 20°C. Below pH 7.2 the oxygen affinity of the polymer appears variable. The haemoglobin exhibits moderate co-operativity, $h = 1.8 \pm 0.3$ (S.D.) at 20°C, which is independent of pH in the range 6.8–8.0. Oxygen-equilibrium studies were also performed on Fraction III from Fig. 7. Fraction III binds oxygen reversibly with a P_{50} of 1.70 kPa (12.8 mmHg) and $h = 1.7$ at pH 8.0 and 20°C. Measurements of absorbance spectra before and after oxygen-equilibrium experiments showed that little methaemoglobin was formed.

Discussion

The erythrocytes of the clam *Barbatia* contain a remarkable intracellular haemoglobin. The protein is extremely large for an intracellular haemoglobin, with a mol.wt. of 430000 by gel chromatography, a value that is consistent with an $s_{20,w}$ of 13.6 S. This is by far the largest intracellular haemoglobin molecule so far reported, with the exception of the fibrous strands exhibited by haemoglobin S. The presence of some 220000-mol.wt. material suggests that the 430000-mol.wt. polymer is unstable and tends to dissociate into approximately one-half molecules. Isolated 220000-mol.wt. material does not appear to re-associate into 430000-mol.wt. material, which suggests that the relationship between 430000- and 220000-mol.wt. fractions is not a simple association–dissociation equilibrium. Although one major haemoglobin peak is resolved by isoelectric focusing of the polymer, some polydispersity is evident. The polydispersity may be explained by the polymer's instability and dissociation properties. This interpretation is strengthened if one considers that urea/gel electrophoresis of globin from intact polymer shows a single polypeptide band. Thus the haemoglobin as well as its subunits appear to be homogeneous.

There is a second haemoglobin in the erythrocytes of *Barbatia*, a tetrameric molecule. Its structure will be presented elsewhere; however, the subunits of the tetramer are electrophoretically distinguishable from the polymer described in the present paper, both with respect to size and charge (N. P. Grinich & R. C. Terwilliger, unpublished work).

Although the polymer contains 1 mol of haem per 16000 g of protein, the smallest subunit produced by a number of harsh denaturing procedures has a mol.wt. of 32000 as analysed by sodium dodecyl sulphate/slab-gel electrophoresis and 37000 mol.wt. by Sephacryl S-200 chromatography in 6 M-guanidine hydrochloride with reducing agent. If the intact haemoglobin is digested with the proteinase subtilisin before sodium dodecyl sulphate slab-gel electrophoresis, a major staining band corresponding to 16500 mol.wt. is observed. This value resembles the molecular weight of a typical haemoglobin subunit. These results suggest that the 32000-mol.wt. subunit may be constructed of two covalently linked oxygen-binding domains. The digested haemoglobin also shows three minor bands with molecular weights between those of the intact subunit (32000) and the putative domain (16500). These minor bands may correspond to polypeptide fragments produced by enzymic cleavage of the subunit at sites other than the putative covalent link connecting the two domains. The results, however, strongly suggest that the 32000-mol.wt. subunit is most susceptible to digestion by the non-specific proteinase subtilisin at a specific region of the polypeptide in accord with the domain hypothesis.

Chromatography by Sephadex G-100 of the digested haemoglobin yields a 30000-mol.wt. haemoglobin-containing fraction. When treated with sodium dodecyl sulphate in the presence or absence of reducing agent, this fraction can be dissociated to a 16500-mol.wt. polypeptide. A probable explanation for this result is that the covalent bond linking the domains is cleaved by the enzyme, but the fragments either retain a dimeric configuration until denatured by sodium dodecyl sulphate or aggregate to that state. Gel filtration of the digested haemoglobin also yielded a 57000-mol.wt. haem-containing fraction; sodium dodecyl sulphate/slab-gel electrophoresis of this fraction showed mostly 16500 mol.wt. (with some 32000-mol.wt.) material. This 57000-mol.wt. fraction of the digested haemoglobin probably is a tetramer of cleaved 16500-mol.wt. polypeptides or possibly some of it may be dimers of undigested subunits. The void-volume fraction of the digested haemoglobin consists of the 32000-mol.wt. subunit, which would be consistent with undigested polymer.

Barbatia intact haemoglobin polymer has a very low oxygen affinity compared with other molluscan haemoglobins (Read, 1966). The moderate co-opera-

tivity of the haemoglobin is independent of pH, and no Bohr effect is apparent between pH 7.2 and 8.0. The isolated 30000-mol.wt. subtilisin-generated haemoglobin fraction also combines reversibly with oxygen. The oxygen affinity of this cleaved, but undissociated, dimer is greater than that of the intact haemoglobin; however, the co-operativity exhibited by this digested material is similar to that of the native haemoglobin. The oxygen-equilibrium properties of the uncleaved 32000-mol.wt. subunit are not known. The main significance that can be attributed to the oxygen-binding studies of the digested material is that the protein can be cleaved into 16000-mol.wt. oxygen-binding fragments. Furthermore these fragments exist in a dimeric state in which some co-operativity is expressed. The question of whether this aggregation is similar to that present in the undigested covalently linked subunit cannot be answered at present.

The subunit structure, a linear series of oxygen-binding domains, for *Barbatia* haemoglobin is similar to that reported for some other invertebrate haemoglobins, all of which are extracellular polymers. These include the extracellular haemoglobins of the branchiopod crustacean *Lepidurus* (Dangott & Terwilliger, 1979), the planorbid snails (Terwilliger *et al.*, 1976; Wood & Gullick, 1979) and the clams *Cardita* and *Astarte* (Terwilliger & Terwilliger, 1978*a*,*b*). The subunit structure of *Barbatia* polymeric haemoglobin, although resembling the domain model suggested for these extracellular haemoglobins, is the first intracellular haemoglobin so far described with this structure.

Several of the arcid bivalves possess intracellular tetrameric and dimeric haemoglobins (Sasakawa & Satake, 1967; Ohnoki *et al.*, 1973; Furuta *et al.*, 1977; Djangmah *et al.*, 1978). *Barbatia* contains a tetrameric haemoglobin, but lacks the typical arcid dimer (N. P. Grinich & R. C. Terwilliger, unpublished work). Instead, *Barbatia* erythrocytes contain a unique high-molecular-weight haemoglobin with this unusual 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, one possibility suggested for other large domain structures (Terwilliger & Terwilliger, 1978*a*). In this regard, sequence studies comparing the domain of *Barbatia* with the polypeptides of other arcid dimers would be interesting. The polymeric haemoglobin found in the erythrocytes of *Barbatia* appears to be an excellent protein for further study of domain structure and synthesis.

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