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Multiple Haemoglobins of the Bivalve Mollusc *Anadara trapezia*

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Abstract

The respiratory proteins of the invertebrate marine mollusc *A. trapezia* have been separated by a combination of gel filtration and ion-exchange chromatography. There is a major tetrameric haemoglobin, HbI, of molecular weight approximately 67 000 and two minor polymorphic haemoglobins HbIIa and HbIIb that are dimeric with molecular weight approximately 31 000, in agreement with previous reports in the literature.

The tetrameric haemoglobin contained α- and β-globin chains that were separated by ion-exchange chromatography in 8 M urea–thiol buffers. The amino acid compositions of the chains showed considerable differences. The amino-terminal residues of these globin chains appear to be blocked but *C*-terminal residues of leucine and alanine were released by carboxypeptidase A digestion.

Both HbIIa and HbIIb contain identical subunits with prolyl amino-terminal residues, while alanine appeared to be the *C*-terminal residue from the rate of liberation by carboxypeptidase A digestion. The amino acid compositions for the two polymorphic globins showed differences only in lysine, aspartic acid and glycine. The *N*-terminal sequence of both HbIIa and HbIIb globin chains was Pro-Ser-Val-Glu-Asp-Ala-Ala-Thr-Leu-Lys.

Introduction

A specialized respiratory protein when present in molluscs is usually haemocyanin. Members of the family Arcidae, however, possess haemoglobin and members of the subfamily Anadarinae are widely distributed in the Indo-Pacific area.

The haemoglobin is found in haemocoelic nucleated erythrocytes and an intracellular haemoglobin of *Anadara broughtonii* (former nomenclature *A. inflata*) was first crystallized by Yagi *et al.* (1957), who reported that it appeared homogeneous on moving boundary electrophoresis and ultracentrifugal analysis. The erythrocytes of Arca, however, contain several haemoglobins as first reported by Yagi *et al.* (1957) and later confirmed (Manwell 1963; Komano 1967; Nicol and O'Gower 1967; Sasakawa and Satake 1967).

The haemoglobins were separated by chromatography on DEAE-cellulose (Komano 1967; Sasakawa and Satake 1967) or by electrophoresis on cellulose acetate (Nicol and O'Gower 1967). The latter authors investigated *Anadara trapezia*, one of four species of the genus that occur around Australian shores and are found in the quieter waters of most bays, inlets and estuaries along the east coast.

The haemolysate of *A. trapezia* was made up of two components designated HbI for the major component, and HbII for the minor component, which was demonstrated to occur in populations in different genetically controlled forms, called HbIIa

and HbIIb, consistent with one pair of alleles. It has been reported (Dixon 1976) that animals possessing one of the polymorphs has an advantage over those with the other at temperatures between 15 and 25°C.

Japanese workers (Sasakawa and Satake 1967; Ohnoki *et al.* 1973) separated haemoglobins I and II from *A. satowi* by gradient elution from DEAE-cellulose (their nomenclature was the reverse of that of Nicol and O'Gower since it was based on the order of elution from DEAE-cellulose), and characterized the components by molecular weight studies which showed that the molecular weight of the major component was 69 000 and that of the minor component 34 000. The subunit molecular weight was approximately the same in both cases showing HbI [Nicol and O'Gower (1967) nomenclature] to be tetrametric and HbII dimeric, with different chemical structures so that HbI is not formed by association of HbII molecules. Ohnoki *et al.* (1973) also showed that Yagi *et al.* (1957) had crystallized HbI because they purified it by repeated salting-out with 52–66% (v/v) of 3·5 M phosphate buffer, and HbII would remain soluble and be removed by this procedure.

Further studies on the subunit structure using haemoglobins from *A. broughtonii* (Furuta *et al.* 1977) showed the presence of different α- and β-globins in the tetrameric HbI, which showed blocked amino terminal residues and leucine *C*-terminal residues. The dimer HbII had an *N*-terminal prolyl residue and a leucine *C*-terminal residue. No resolution of HbII corresponding to the polymorphic forms HbIIa and HbIIb was reported by the Japanese workers.

This paper reports studies on the purification of haemoglobins HbI, HbIIa and HbIIb and their globin chains from *A. trapezia*. Amino acid compositions and terminal group analyses are reported together with the amino terminal sequence of HbIIa and HbIIb.

Materials and Methods

Collection and Treatment of Blood Samples

A. trapezia specimens were collected at low tide from bays and estuaries in the Sydney area, particularly Gunnamatta Bay, Port Hacking, and Towra Point in Botany Bay. Haemolymph from the pallial sinuses was drained through glass wool into ice-cooled containers, and the pooled sample conveyed to the laboratory. The red cells were packed by centrifugation at 1500 *g* for 10 min, washed twice with 1% (w/v) NaCl and lysed by shaking with ice-cold water containing 0·1 vol. CCl_4. The haemolysate was centrifuged at 25 000 *g* and 2°C to remove cell debris and, after dialysis against cold water, concentrated by pressure dialysis (Berggard 1961). The haemoglobins were converted to the carbon monoxide form and dialysed against buffer required for chromatography.

Chromatography of Haemolysates

Gel filtration was done on Sephadex G100 columns (120 by 2·8 cm) equilibrated with 0·05 M sodium phosphate, pH 7. Ion-exchange chromatography utilized CM-Sephadex C50 columns (20 by 2·8 cm) equilibrated in 0·05 M phosphate buffer, pH 6·0, and developed with a series of buffers of pH 6·0, 8·0 and 9·0 to separate haemoglobins I, IIa and IIb respectively. Haemoglobin I and non-haem contaminant proteins were eluted in the pH 6·0 buffer and a linear gradient from 0 to 0·1 M Na^+ in the buffer resulted in both haemoglobins IIa and IIb being eluted together.

DEAE-Sephadex A50 columns (20 by 2·8 cm) were used equilibrated with 0·05 M Tris-HCl, pH 7·4, and a linear gradient device, 100 ml each chamber, to 0·05 M imidazole-HCl, pH 6·0, for separation of unadsorbed haemoglobins IIa plus IIb from haemoglobin I. Separation of this mixture after dialysis utilized a similar column equilibrated at pH 9·4 with a pH gradient to 8·6 using 0·05 M Tris-HCl buffers.

The fractions were examined by Cellogel electrophoresis as described by Nicol and O'Gower (1967) and Fisher and Thompson (1979).

Preparation and Fractionation of Globins

The globin chains were prepared by acid–acetone precipitation as previously described by Fisher and Thompson (1979).

Fractionation of globin I chains was done on a column (20 by 2 cm) of DEAE-Sephadex A50 using buffer containing 8 M urea–0·05 M Tris–0·05 M mercaptoethanol adjusted to pH 8·5 with HCl. A linear gradient of NaCl from 0 to 0·1 M was used to separate the chains, which were carboxymethylated before recovery (Thompson *et al.* 1969). Alternatively, the globins could be fractionated on CM-Sephadex C50 columns using buffer containing 8 M urea–0·05 M sodium (phosphate)–0·05 M mercaptoethanol, pH 6·5, for equilibration, loading and washing, with development of the column by a similar buffer of pH 7·8 to separate the β- and α-globins.

Electrophoresis of samples of the globin fractions used Cellogel and 8 M urea buffers (Nash and Thompson 1974).

Molecular Weight, Amino Acid Analyses and Sequence Determinations

The methods for estimation of molecular weight by ultracentrifugation and polyacrylamide disc electrophoresis in the presence of sodium dodecyl sulfate, amino composition after acid or alkaline hydrolysis, peptide mapping and amino acid sequence by the dansyl–Edman procedure were substantially the same as previously described (Thompson *et al.* 1969; Air and Thompson 1969, 1971; Beard and Thompson 1971; Moon and Thompson 1971; Nash and Thompson 1974).

For carboxypeptidase digestions 5 mg of each globin in 3 ml 1% (w/v) ammonium bicarbonate was treated with 50 μg carboxypeptidase solution (Worthington, DFP-treated) at 37°C. Samples were withdrawn at 30-min intervals for amino acid analysis.

Results

Haemoglobin Purification

Cellogel electrophoresis at pH 9·2 of the total haemolysates made from pooled samples gave results similar to those reported by Nicol and O'Gower (1967). Densitometric scan of the cleared gels showed the ratio of the major to the minor haemoglobin zones to be approximately 65 to 35, similar to that reported by Ohnoki *et al.* (1973) for *A. satowi.* The major zone is called haemoglobin I. There are two polymorphic forms in the minor zone, called haemoglobins IIa and IIb, which are separated best by Cellogel electrophoresis at pH 9·5. The HbIIa and HbIIb occur in this zone in the ratio of approximately 1 to 4. Although Nicol and O'Gower (1967) and Collett and O'Gower (1972) have reported no major contaminants of the haemoglobins in the red cells, with large collections it proved difficult to avoid some contamination from non-haem proteins. For this reason the haem group should be retained as an aid during the purification as long as possible.

The spectral properties show a great similarity between these intracellular haemoglobins and those from other sources, the cyanomethaemoglobin from the pooled material showing maxima at 540 and 412 nm.

The haemoglobins are relatively unstable and are best stored in the carbonmonoxy form. The heat stabilities of HbI and HbIIa+IIb were almost identical with over 90% denaturation at 60°C within 10 min. In this respect they are far less stable than human haemoglobin (7% in 10 min). In 0·1 M Tris–17% (v/v) isopropanol at 37°C the *Anadara* haemoglobins are similarly much less stable than human haemoglobin (6% denaturation in 10 min), but the dimeric haemoglobins IIa+IIb showed greater stability than HbI (43 and 86% respectively).

The fractionation of the haemoglobins of *A. trapezia* red cell haemolysate by gel filtration is shown in Fig. 1*a*. The main haemoglobin fractions are HbI in peak 2 and HbIIa+IIb in peak 3, the other peaks containing predominantly non-haem proteins.

Isoelectric focusing in a stabilized pH gradient produced by Ampholine carrier ampholytes (0·5% (w/v), pH 3–10) at 0°C for 72 h gave the isoelectric points as HbI 4·95; HbIIa 9·10; and HbIIb 9·35.

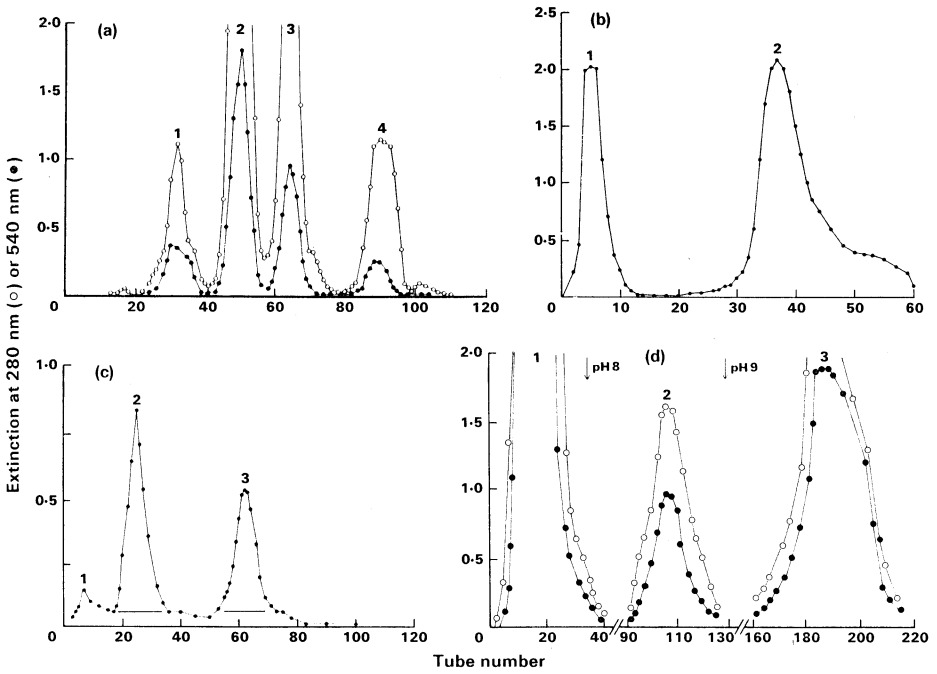

Fig. 1. (*a*) Gel filtration of the *A. trapezia* red cell haemolysate on a column of Sephadex G100 (2·8 by 120 cm) equilibrated and eluted with 0·05 M phosphate, pH 7·0. Fraction size 5 ml. Haemoglobins I and IIa+IIb are in peaks 2 and 3 respectively. The absorption at 540 nm in peaks 1 and 4 are attributed to aggregated and degraded haem proteins.
(*b*) Chromatography of haemoglobins of *A. trapezia* red cell haemolysate on a column of DEAE-Sephadex A50 (1·8 by 20 cm) by linear gradient elution at room temperature. The unadsorbed fraction containing haemoglobins IIa+IIb was washed off with 100 ml starting buffer, 0·05 M Tris-HCl, pH 7·4, before commencing the gradient, 100 ml each chamber, to the limit buffer, 0·05 M imidazole-HCl, pH 6·0. Fraction size 5 ml. The second peak contained haemoglobin I and non-haem contaminant proteins.
(*c*) Separation of haemoglobins IIa and IIb of *A. trapezia* on a column (2·8 by 20 cm) of DEAE-Sepahdex A50 equilibrated with 0·05 M Tris-HCl, pH 9·4. A pH gradient between 9·4 and 8·6 was established with 150 ml per chamber. Fraction size 3 ml. Haemoglobin IIb eluted before haemoglobin IIa. The tubes pooled to provide each haemoglobin are shown as bars.
(*d*) Separation of the haemoglobins of *A. trapezia* haemolysate on a column (8 by 40 cm) of carboxymethyl-Sephadex C50 equilibrated with 0·05 M sodium phosphate, pH 6·0. A 10% (w/v) solution (120 ml) was loaded and washed with a further 120 ml of pH 6 buffer. The buffer pH was increased to pH 8 and pH 9 as indicated by the arrows. Fraction size approximately 6 ml. Peak 1 contains haemoglobin I plus non-haem proteins, peaks 2 and 3 contain haemoglobins IIa and IIb respectively.

When the haemolysate is chromatographed on DEAE-Sephadex at pH 7·4 (Fig. 1*b*) the haemoglobins IIa plus IIb are not adsorbed while the HbI is bound to the absorbent and eluted together with non-haem contaminant proteins and degraded haem proteins present in aged samples by a pH gradient to pH 6. This result is similar to that reported by Ohnoki *et al.* (1973).

The mixture of haemoglobins IIa and IIb from this DEAE-Sephadex column can be fractionated on a column of DEAE-Sephadex equilibrated and loaded at pH 9·4. The haem proteins are bound and eluted with a pH gradient from 9·4 to 8·6 (Fig. 1*c*). The HbIIb elutes before the HbIIa.

With CM-Sephadex at pH 6 (Fig. 1*d*), the haemoglobin I plus non-haem contaminant proteins are not absorbed. The haemoglobin IIb can be eluted with buffer of pH 8, while HbIIa is eluted at pH 9.

Because of the limited stability of the haemoglobins the method of choice for purification may preferably purify one of the components at the expense of the other components in the interest of speed of separation.

The combination of appropriate ion-exchange chromatography and gel filtration has yielded pure samples of HbI, HbIIa and HbIIb.

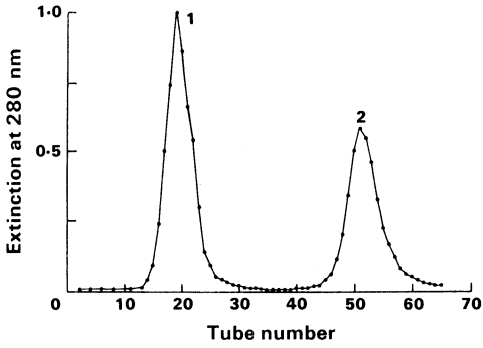

Fig. 2. Separation of the globin chains from haemoglobin I of *A. trapezia* on a column (2 by 20 cm) of DEAE-Sephadex A50 at room temperature. The column was equilibrated and developed with buffer containing 8 M urea–0·05 M Tris-HCl–0·05 M mercaptoethanol, pH 8·4, and loaded with 100 mg globin dissolved in the starting buffer. A linear gradient from 0 to 0·1 M NaCl in the buffer, 100 ml each chamber, was used. Fraction size 3 ml. Peak 1 contained α-globin and peak 2 β-globin.

Molecular Weight Determinations

Molecular weight values obtained for *A. trapezia* haemoglobins and globin chains by standard methods of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, gel filtration on Sephadex G100 in 8 M urea buffer and analytical ultracentrifugation using sedimentation velocity are shown in the following tabulation:

Sample	SDS polyacrylamide gel electrophoresis	Gel filtration	Sedimentation velocity
Haemoglobin I		64 000	67 300
Haemoglobin IIa+IIb		29 000	31 000
Globin I α-chain	17 000	15 100	
Globin I β-chain	16 000		
Globin IIa+IIb	15 200	14 700	

The sedimentation velocity values, as well as the gel filtration results, indicate a tetrameric molecular weight of approximately 67 000 for HbI and a dimeric molecular weight of approximately 31 000 for *Anadara* haemoglobins HbIIa and HbIIb. The subunit molecular weights of approximately 15 000–16 000 are indicated by the SDS-polyacrylamide gel electrophoresis results, as well as by gel filtration in 8 M urea buffers in comparison with standard proteins. These results are in agreement with those reported for *A. satowi* by Ohnoki *et al.* (1973), and for *A. broughtonii* by Furuta *et al.* (1977).

Globin Chain Purification

Cellogel electrophoresis in 8 M urea buffers showed the presence of dissimilar subunits only in globin I, the best separation being observed at pH 7·2.

The globin chains were separated by chromatography in 8 M urea buffer with a salt gradient either on DEAE-Sephadex (Fig. 2) or CM-Sephadex. The α-chain emerged first from DEAE-Sephadex columns but last from CM-Sephadex columns, where the designation 'α' and 'β' was made on the electrophoretic mobility of the chains relative to human α- and β-globin chains. The α-globin has the lowest mobility in agreement with the results of Furuta *et al.* (1977) for *A. broughtonii* globins. Any contamination from non-haem proteins could be avoided by purifying the haemoglobin carefully before conversion to globin, or by gel filtration of the separated chains on Sephadex G100 using 8 M urea buffers, the globin being more retarded.

Table 1. Amino acid composition of globin chains from *A. trapezia*

Samples of the carboxymethylated globins were hydrolysed with 6 M HCl containing 0·1% (w/v) phenol and 0·05% (w/v) mercaptoethanol in sealed evacuated tubes at 110±2°C. Mean extrapolated or maximum values are given as moles per mole of protein relative to the phenylalanine content, and are corrected for losses during hydrolysis and incomplete hydrolysis by extrapolation or selection of 24-, 48- and 72-h values. The most probable value is given in parentheses. Tryptophan was determined by alkaline hydrolysis

Amino acid	Globin I α-chain	Globin I β-chain	Globin IIa	Globin IIb
Lys	10·8 (11)	7·9 (8)	13·3 (13)	14·0 (14)
His	1·8 (2)	1·8 (2)	2·6 (3)	2·7 (3)
Arg	7·9 (8)	7·6 (8)	3·8 (4)	4·0 (4)
CMCys	1·8 (2)	1·0 (1)	0·9 (1)	1·1 (1)
Asp	18·3 (18)	20·4 (20)	20·8 (21)	20·2 (20)
Thr	7·7 (8)	6·8 (7)	7·0 (7)	7·1 (7)
Ser	11·1 (11)	12·2 (12)	7·9 (8)	8·2 (8)
Glu	8·0 (8)	10·9 (11)	10·0 (10)	10·4 (10)
Pro	2·7 (3)	1·3 (1)	2·0 (2)	2·0 (2)
Gly	12·2 (12)	9·2 (9)	11·5 (12)	10·8 (11)
Ala	18·0 (18)	18·3 (18)	16·1 (16)	16·0 (16)
Val	11·7 (12)	14·7 (15)	10·7 (11)	10·6 (11)
Met	3·5 (4)	4·5 (5)	5·5 (6)	5·8 (6)
Ile	7·4 (8)	4·8 (5)	6·8 (7)	7·2 (7)
Leu	15·0 (15)	18·1 (18)	12·6 (13)	12·8 (13)
Tyr	3·6 (4)	2·1 (2)	2·7 (3)	2·5 (3)
Phe	5·0 (5)	6·0 (6)	6·0 (6)	6·0 (6)
Trp	2·7 (3)	2·2 (2)	1·9 (2)	1·9 (2)
Total	152	150	145	144

Amino Acid Composition

The amino acid composition of the purified globin chains from HbI, HbIIa and HbIIb are shown in Table 1. There are considerable differences between the globin I α- and globin I β-chains. The differences between globins IIa and IIb are only in the lysine, glycine and aspartic acid contents, in agreement with the polymorphic nature

of these proteins (Nicol and O'Gower 1967). The results of Furuta *et al.* (1977) for *A. broughtonii* globins show significant differences from the values presented here. Peptide mapping of the tryptic digests of globin IIa and globin IIb revealed only one obvious difference in the maps with a change in mobility of an acidic peptide at pH 6·4.

Terminal Groups

There was no detectable amino terminal residue in the globin I α- or globin I β-chains by the dansyl method. Both globin IIa and globin IIb gave dansyl-proline, suggesting an identical *N*-terminal sequence. The tryptic peptides of globins IIa and IIb were eluted from a peptide map and the *N*-terminal residues examined by the dansyl method. Only one acidic spot at pH 6·4, in an identical position on each map, gave dansyl–proline, presumably the *N*-terminal peptide. The sequence of this peptide was determined by the dansyl–Edman procedure as

Pro-Ser-Val-Glu-Asp-Ala-Ala-Thr-Leu-Lys.

The carboxyl terminal residues released by carboxypeptidase A were similar for globin IIa and globin IIb with alanine being released most rapidly (0·4 mol/mol in 2 h at 37°C) followed by valine and leucine (approximately 0·2 mol/mol). With globin I α-chain leucine was released most rapidly for carboxypeptidase A (0·2 mol/mol in 2 h at 37°C) followed by alanine, whereas for globin I β-chain this order was reversed with alanine being released more rapidly than leucine (approximately 0·2 mol/mol in 2 h at 37°C).

In view of the poor yield of amino acids in the carboxypeptidase A digestions and the similarity in our results to those of Furuta *et al.* (1977) for *A. broughtonii*, no firm conclusions can be drawn for the *C*-terminal residues or sequences. These authors reported that every chain contained alanine, leucine and valine residues adjacent to the carboxyl termini with the β-globin giving a slightly different digestion profile to α-globin or HbII, yet all chains gave leucine on hydrazinolysis. Only for globin I α-chain of *A. trapezia* has the *C*-terminal leucine been confirmed by hydrazinolysis and amino acid sequence studies (Como and Thompson, unpublished data).

Discussion

There is now abundant evidence from X-ray crystallographic analysis and amino acid sequence studies that haemoglobin and myoglobin evolved from a common polypeptide precursor gene. The myoglobin tertiary structure first shown in sperm whale myoglobin (Kendrew *et al.* 1960) is also common to haemoglobin from phylogenetically remote animals and the evidence from leghaemoglobin (Vainshtein *et al.* 1975) indicates that the myoglobin structure was present in respiratory proteins of organisms which existed between $1 \cdot 3 \times 10^9$ and $1 \cdot 5 \times 10^9$ years ago, when the plant and animal kingdoms had their origins.

Both myoglobins and haemoglobins occur in invertebrates. The amino acid sequences have been described for six invertebrate globins representative of molluscs, arthropods and annelids. Myoglobin sequences have been published for the two gastropod molluscs *Aplysia limacina* (Tentori *et al.* 1973) and *Busycon canaliculatum* (Bonner and Laursen 1977). Imamura *et al.* (1972) established the sequence of the

monomeric haemoglobin component from the annelid *Glycera dibranchiata*, and of 11 haemoglobin components from the insect *Chironomus thummi thummi* III, IIβ and VIIIβ have been sequenced (Buse *et al.* 1969; Kleinschmidt and Braunitzer 1976; Sladic-Simic *et al.* 1977). These invertebrate sequences vary in length between 136–147 residues. X-ray analysis of *Glycera* sp. haemoglobin and *Chironomus thummi thummi* haemoglobin III indicates conformational similarity of these molecules with vertebrate myoglobin and haemoglobin (Huber *et al.* 1968; Hendrickson and Love 1971; Padlan and Love 1974).

In the *A. trapezia* major haemoglobin, HbI, approximately 65% has been characterized in the present study as tetrameric, with two different globin chains of approximately 152 residues in the $\alpha_2\beta_2$ pattern which is characteristic of vertebrate haemoglobins. In addition, there is a minor haemoglobin which is dimeric with identical subunits which can occur in two polymorphic forms of approximately 144 residues. Both haemoglobin IIa and haemoglobin IIb have the same *N*-terminal sequence Pro-Ser-Val-Glu-Asp-Ala-Ala-Thr-Leu-Lys, and the same *C*-terminal residues, alanine, released by carboxypeptidase A.

Read (1966) proposed that at least 21 species of bivalves possess haemoglobin, often in multiple forms. In the case of other species of *Anadara*, apart from *A. trapezia* (Nicol and O'Gower 1967), multiple forms have been reported for *A. satowi* (Ohnoki *et al.* 1973) and *A. broughtonii* (Yagi *et al.* 1957; Furuta *et al.* 1977). Other closely related species, *A. granosa*, *A. maculosa*, *A. transversa*, *A. hemidemos*, *A. ovalis and Noetia ponderosa* (Manwell 1963; Collett and O'Gower 1972; O'Gower and Matthewson, personal communication) also show multiple haemoglobins. The overall pattern of haemoglobins of *Anadara* spp., their terminal residues and amino acid compositions seem to be remarkably similar, any differences being the likely result of evolutionary divergence in the different species of the genus. The natural occurrence of a stable haemoglobin with a dimer structure is unusual.

Multiple haemoglobins are also a feature of lower vertebrates (Riggs 1970; Gillen and Riggs 1972; Powers 1974) with fish haemoglobins often being polymorphic (Fyhn and Sullivan 1974; Powers 1974). It was suggested by Riggs (1970, 1979) that the possession of multiple haemoglobins conferred a selective advantage on an animal species, and throughout the lower vertebrate and invertebrate groups it does seem a widespread mechanism of adaptation to variable environmental factors. Functional differences in multiple haemoglobins are well documented for fish haemoglobins (Riggs 1979) and have been demonstrated in haemoglobins from insects (Braun *et al.* 1968), echinoderms (Bonaventura *et al.* 1976), annelids (Seamonds and Forster 1972; Wells and Dales 1976), and molluscs (O'Gower and Nicol 1968; Collett and O'Gower 1972; Dixon 1976).

Further characterization of the globin chains of *A. trapezia* by amino acid sequence studies may assist in relating their structure to their differences in function and evolution.

Acknowledgments

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