The structure of *Artemia* sp. (brine shrimp) haemoglobins*

Purification of a structural unit to homogeneity

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The extracellular haemoglobins (M_r 260000) of the brine shrimp *Artemia* sp. were cleaved by limited digestion with subtilisin. Structural units of M_r 16000, which can bind dioxygen reversibly, were isolated. Analysis of the 16000- M_r fraction (E) reveals the presence of a limited number of structural units. A single type of structural unit, E₁ (M_r 15800; pI 4.8), was purified to homogeneity and characterized.

The primary structure of invertebrate globins (myoglobins and haemoglobins) is only available for a limited number of species (Annelida: *Lumbricus*, *Tylorhynchus*, *Glycera*; Mollusca: *Aplysia*, *Anadara*, *Buscon*, *Cerithidea*; Arthropoda: *Chironimus*).

All the sequenced polypeptides belong to the low- M_r globin-chain types, having an M_r of about 16000 and binding one haem group (Imamura *et al.*, 1972; Bonner & Laursen, 1977; Como & Thompson, 1980; Fisher *et al.*, 1984; Furuta & Kajita 1983; Garlick & Riggs, 1982; Suzuki *et al.*, 1982; Goodman *et al.*, 1983; Takagi *et al.*, 1983; Shikama *et al.*, 1982).

Their sequence alignment show a definite relationship with their vertebrate counterparts (Dayhoff, 1978; Fasman, 1976; Goodman, 1981). This is confirmed by X-ray analysis of *Chironimus* and *Glycera* haemoglobins, which clearly shows the myoglobin fold (Padlan & Love, 1968; Huber *et al.*, 1971). In contrast, no structural information is available on the invertebrate high- M_r globin-chain types. These globin chains, with M_r values from 32000 to 300000, and binding two to sixteen haem groups, are found in molluscs and arthropod extra-

cellular haemoglobin. They are, like the haemocyanins, built up as polymers of structural and functional units resembling the low- M_r globin-chain types (Chung & Ellerton, 1979; Wood, 1980; Geelen *et al.*, 1982; Moens *et al.*, 1984a).

Studies on globin evolution and phylogeny are very detailed for vertebrate, but incomplete for invertebrate, globins (Kimura, 1968, 1969; King & Jukes, 1969; Goodman *et al.*, 1974, 1975; Goodman, 1981). However, globin evolution must have been more dramatic during invertebrate than during vertebrate evolution, where only a refinement of the oxygen carrier occurred.

The present view on globin evolution, based on protein and gene structure, suggests the evolution from a common ancestor (Hunt *et al.*, 1978; Jensen *et al.*, 1982). However, the extracellular haemoglobins of the invertebrates with their extraordinary architecture cannot be accommodated into this model (Wood, 1980; Chung & Ellerton, 1979; Hendrickson, 1979).

As a working hypothesis, the aberrant structures of the invertebrate haemoglobins can be considered as adaptations of the ancestral globin (M_r ~16000)/globin-gene complex to the specific invertebrate situation. The lack of an 'erythrocyte' cell type to store the oxygen carrier forces the extracellular haemoglobin to acquire a high M_r in order to avoid physical loss of the pigment. This high M_r was attained by the aggregation of low- M_r globin chains by disulphide interaction, as found in the Annelida, or by the covalent linkage of the ancestral low- M_r polypeptides into globin chains with multiple structural units, as found in the Mollusca and Arthropoda.

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; DMSO, dimethyl sulphoxide; DABITC, 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate; SDS, sodium dodecyl sulphate; Hb II, haemoglobin II; PITC, phenyl isothiocyanate.

* The present paper is paper III in a series of papers on this topic. Paper I is Geelen *et al.* (1982) and paper II is Moens *et al.* (1984a).

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To prove this hypothesis, more information on invertebrate globin chains is essential.

In the present paper the purification of a single structural unit isolated from the high- M_r globin chains of the brine shrimp *Artemia* sp. is described (Moens & Kondo, 1977, 1978; Wood *et al.*, 1981; Geelen *et al.*, 1982; Moens, 1982; Wolf *et al.*, 1983; Moens *et al.*, 1984a,b).

Materials and methods

Isolation of a structural unit

Artemia sp. (Salines du Midi, Le Grau du Roi, France) haemoglobins were prepared as described by Moens & Kondo (1978) and Geelen *et al.* (1982). Purified haemoglobins in 40 mM-glycine/NaOH (pH 10.5)/10 mM-EDTA were digested with subtilisin at an enzyme/substrate ratio of 1:50 at 25°C for 60 min. After stopping the reaction with PMSF (1 mg of Hb/ml in 100 μ l of DMSO) the digestion mixture was chromatographed on a Sephacryl S-200 (superfine) column and the 15000–20000- M_r polypeptides were collected (Moens *et al.*, 1984a).

Elimination of excess subtilisin

After adjusting the pH to 7.6 the 15000–20000- M_r fraction was loaded on to a Whatman DE 52 column (3 cm $\times$ 10 cm) equilibrated in 0.2 M-glycine buffer (pH 7.6)/KCN (100 mg/litre) (Schroeder & Huisman, 1981).

After binding had taken place, the column was extensively washed with starting buffer, after which the structural units were eluted in a small volume with the same buffer containing 250 mM-NaCl. Subtilisin activity was measured by using Hide Powder Azure as a synthetic substrate (James, 1978).

Isoelectric focusing

Preparative isoelectric focusing of the structural units was performed in a Sephadex G-75 (superfine) layer (21 cm $\times$ 25 cm $\times$ 0.15 cm), containing 4 M-urea and 2% (v/v) Ampholines (pH range 4.0–6.5) as described by Radola (1973, 1974) and Frey & Radola (1982).

Material was loaded on a line 5 cm from the cathode site at the rate of 10 mg/cm and focusing was performed initially at 3 W for 1 h, followed by 4 h at 10 W. After focusing, the layer was scanned at 420 nm by using a Zeine soft-laser densitometer. The gel containing the bright red bands was scraped off with a spatula, packed as a small column and the protein material eluted with 50 mM-Tris/HCl (pH 7.5)/4 M-urea.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis under denaturing conditions in the presence of SDS or acid

urea was performed as described by Panyim & Chalkley (1969) and Laemmli (1970).

Polyacrylamide-gel electrophoresis under 'native' conditions was performed with a 7.5% (w/v)-polyacrylamide gel in a discontinuous buffer system as described by Waring *et al.* (1970). Two-dimensional separations were performed by the method of O'Farrell (1975). In the first dimension, proteins were focused in 5%-polyacrylamide gels in 2% Ampholines (pH 3–10) at 400 V for 15 h and at 500 V for 1 h. For the second dimension, a 15%-polyacrylamide gel was used (Laemmli, 1970).

Amino acid analysis

Protein samples were hydrolysed in 6 M-HCl containing 4% (w/v) thiodiglycolic acid in evacuated sealed tubes for 24 h at 110°C. Amino acid analysis was performed with a Jeol JLC 6AH

Fig. 1. Separation by gel filtration and analysis on polyacrylamide gels

(a) Separation by gel filtration of the fragment mixture resulting from limited subtilisin digestion of intact *Artemia* haemoglobins. Digestion was performed at an enzyme-to-substrate ratio of 1:50 for 60 min at 25°C. The fragments were separated by chromatography on a Sephacryl S-200 column equilibrated in 40 mM-glycine/NaOH (pH 10.5)/2.6 mM-PMSF at a flow rate of 66 ml/h and the fractions pooled as indicated. (b) Analysis on SDS/15%-polyacrylamide gel as described by Laemmli (1970) of fraction E. M, M_r markers; E, fraction E from (a). (c) Analysis on 7.5%-polyacrylamide gel as described by Waring *et al.* (1970) of fraction E. M, human haemoglobin (Hb) and albumin (Alb) were used as markers; E, fraction E from (a).

amino acid analyser as described by Spackman *et al.* (1958).

Sequence analysis

The determination of the *N*-terminal sequence was carried out on a micro scale (10 nmol) with DABITC in the double-coupling method described by Chang (1978) and Chang *et al.* (1978).

Other methods

Other techniques (subtilisin digestion, protein determination, oxygen binding and immunodiffusion) were as described by Geelen *et al.* (1982), Wolf *et al.* (1983) and Moens *et al.* (1984a).

Results

Isolation and purification of a structural unit

The 15000–20000- M_r fraction (Fig. 1a) isolated from subtilisin-digested *Artemia* haemoglobin by Sephacryl S-200 chromatography, representing single structural units, is heterogeneous in M_r and charge (Figs. 1b and 1c) (Moens *et al.*, 1984a). This is in agreement with similar results from other invertebrate haemoglobins (Terwilliger & Terwilliger, 1977, 1978; Terwilliger *et al.*, 1977; Dangott & Terwilliger, 1979, 1980, 1981; Wood & Gullick, 1979). This heterogeneity may result from the aspecific proteolytic cleavage of a single type of

structural unit from 'homopolymeric' globin chains or from the existence of several types of structural units within a 'heteropolymeric' globin chain, or both.

The purification of a single structural unit from fraction E to homogeneity is completely dependent on the elimination of traces of subtilisin contaminating this fraction. This was achieved by chromatography on a DE 52 column at pH 7.6, as described by Schroeder & Huisman (1981) (Fig. 2). Under these conditions, the fragments were quickly washed free of proteinase activity and were eluted with 250 mM-salt as a very sharp band, whereas oxidized material was irreversibly bound.

The proteinase-free material was analysed by two-dimensional electrophoresis as described by O'Farrell (1975). Four major fractions, and several minor ones, with M_r 15000–18000 and pI between 6.4 and 7.9 were revealed (Fig. 3 and Table 1).

Separation of the different types of structural units in the 15000–18000- M_r fraction by different chromatographic (high- and low-pressure) and preparative electrophoretic techniques was unsuccessful.

A pure fraction containing a single structural unit type could only be obtained by isoelectric focusing (pH 4.0–6.5) on a Sephadex G-75 (superfine) layer performed by the method of Radola (1973, 1974) and Frey & Radola (1982) (Fig. 4).

Fig. 2. Elimination of subtilisin by ion-exchange chromatography

Fraction E (Fig. 1a) was adjusted to pH 7.6 and loaded on to a Whatman DE 52 column (1.5 cm × 10 cm) equilibrated in 0.2 M-glycine/KCN. After loading, the column was washed with the same buffer and the haemoglobin fragments were eluted with 0.25 M-NaCl in equilibration buffer. Subtilisin activity was measured as described by James (1978).

Fig. 3. Analysis of fraction E by the O'Farrell (1975) technique

Fraction E (Figs. 1 and 2) was analysed in the first dimension by isoelectric focusing on a 5% (w/v)-polyacrylamide gel in a pH 3–10 gradient. A 15% (w/v)-polyacrylamide gel was used (Laemmli, 1970) in the second dimension. Gels were stained with Coomassie Blue R250. (a) Densitometric scanning of a stained one-dimensional gel; (b) alignment of a one- and two-dimensional gel.

The most acidic fraction, E₁, appears as a bright red and sharp band, well separated from the rest. The other bands (E₂–E₅) are less sharp and more reddish-brown, which most likely indicates autooxidation. They appear on a rather diffuse background. The proportions of the five fractions are variable. This is probably due not to the separation conditions but rather to the conditions of the haemoglobin digest.

The five fractions (E₁–E₅) were analysed by one- and two-dimensional polyacrylamide-gel-electrophoresis techniques under native and denaturing conditions (Fig. 5).

Table 1. Analysis of the structural-unit fraction
Fraction E was separated preparatively by isoelectric focusing on a Sephadex G-75 layer under native conditions (Frey & Radola, 1982), after which each fraction was analysed in a two-dimensional system under denaturing conditions as described by O'Farrell (1975). Minor fractions are indicated by italics.

Native conditions (Fig. 4)		Denaturing conditions (Figs. 3 and 5)		
Fraction	pI	Fraction	10 ⁻³ × M _r	(Fig. 3) pI
E ₁	4.8	E _A	15.8	6.40
E ₂	5.16	<i>E_{ab}</i>	15.8	7.00
		E _B	17.2	7.55
		<i>E_{Abc,fg}</i>		
E ₃	5.30	E _C	15.8	7.15
		<i>E_g</i>	17.2	7.95
E ₄	5.50	<i>E_{Abc,B,D}</i>		
		E _C	15.8	7.15
		<i>E_e</i>	15.8	7.45
E ₅	5.62	<i>E_D</i>		
		<i>E_g</i>	17.2	7.95
		<i>E_B</i>		

On SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), two bands with M_r values of 15800 ± 800 (n = 6) and 17200 ± 900 (n = 6) respectively were obtained. E₁ and E₅ clearly contain single bands (M_r 15800 and 17200), whereas E₂, E₃ and E₄ are double (Fig. 5a).

A similar pattern was obtained with acid-urea gels (Panyim & Chalkley, 1969). Separation under native conditions (Waring *et al.*, 1970) reveals four different charge populations. Only E₁ shows a single band (Fig. 5b).

When analysed by the two-dimensional O'Farrell (1975) technique (Fig. 5c), only E₁, as might be expected, is homogeneous, whereas the other fractions seem to be stable aggregates of polypeptides with different M_r values and charges, confirming the results shown in Figs. 5(a) and 5(b) and in Table 1.

Characterization of the structural unit E₁

Fraction E₁, isolated by the above-described method and representing a single type of structural unit, is fairly homogeneous (~90%) (Fig. 5c). The N-Terminal sequence was analysed by using the DABITC/PITC double-coupling method of Chang (1978) and Chang *et al.* (1978).

Six residues were identified unambiguously:

Glu-Arg-Val-Asp-Pro-Ile-

Contaminants were negligible.

Fig. 4. Preparative isoelectric focusing of fraction E

Preparative isoelectric focusing was performed in a Sephadex G-75 (superfine) layer (21 cm $\times$ 25 cm $\times$ 0.15 cm) containing 4M-urea and 2% Ampholines, pH 4–6.5, as described by Frey & Radola (1982). Fraction E (Fig. 2) was loaded at 5 mg/cm and focused initially at 3 W for 1 h, followed by 4 h at 10 W.

Under native conditions E_1 has an M_r of 17400 ± 200 ($n = 5$) and apparent pI 4.8 (Table 1). Under denaturing conditions an M_r of 15800 ± 800 ($n = 6$) and pI 6.4 were determined (Table 1).

E_1 is, like intact Hb II and fraction E, still able to react with an anti-(Hb II) IgG, indicating that they share at least some common antigenic determinants.

The amino acid composition of E_1 shows high contents of aspartic acid, glutamic acid, valine and leucine. Cysteine is absent (Table 2). The amino acid composition of E_1 is quite similar to that of Hb II, confirming that no specific amino acid sequences exist in the intact haemoglobin molecules (Geelen *et al.*, 1982; Moens *et al.*, 1984a).

The determined amino acid composition is compatible with an M_r of about 16000 and 145–150 residues. The isolated structural unit is still able to bind dioxygen reversibly. It has a very high oxygen affinity [$p_{50} = 0.36 \pm 0.045$ mmHg (47.98 ± 5.99 Pa) ($n = 10$)], but, as expected, no cooperativity [$n = 0.95 \pm 0.09$ ($n = 10$)] (Figs. 6a and 6b).

E_1 thus represents not only a structural unit, but also a functional entity from which the intact *Artemia* haemoglobin molecule is built up.

Discussion

As a working hypothesis, the *Artemia* sp. globins can be considered as built up, like other invertebrate globins (Chung & Ellerton, 1979; Ilan & Daniel, 1979; Wood, 1980), by the covalent

linkage of structural units of $M_r \sim 16000$ (Geelen *et al.*, 1982; Moens *et al.*, 1984a). These structural units are equivalent in primary structure and configuration with the classical vertebrate globins. Similar structures are found in the haemocyanin polypeptide chains (Gielens *et al.*, 1977, 1980; Wood, 1980).

Globin chains with multiple functional units may be the product of a gene originated by a series of tandem duplications of an ancestral globin gene.

Such an event has a strong selective advantage during evolution because 'polymerization' of a globin chain increases the M_r of its haemoglobin, protecting it against elimination by excretory processes and lowering the colloid osmotic pressure of the haemolymph in which it is dissolved (Lee & Smith, 1965; Wood, 1980).

The globin chains (α , β , χ) that form the *Artemia* haemoglobins have differing genetic behaviours (Bowen *et al.*, 1977; Sterling & Bowen, 1977) and are slightly different in structure and function (Moens & Kondo, 1978; D'Hondt *et al.*, 1978; Wood *et al.*, 1981). We assume the existence of two to three basically different structural units specific for each globin chain and determining its physiological characteristics.

However, owing to genetic events within the 'multiplied' genes, the structural units and/or the linking regions within a globin chain may show variations in the basic structure of that chain (heteropolymers).

Our results are compatible with this hypothesis. Supposing a random scission of the haemoglobin

Fig. 5. Analysis of fractions E_1 – E_5 by polyacrylamide-gel electrophoresis

(a) SDS/15%-polyacrylamide-gel electrophoresis as described by Laemmli (1970). (b) 7.5%-Polyacrylamide-gel electrophoresis under native conditions (running at pH 9.0) as described by Waring *et al.* (1970). (c) O'Farrell (1975) analysis of fraction E_1 . First dimension: 5%-polyacrylamide gel, pH gradient 3–10; second dimension: SDS/15%-polyacrylamide-gel electrophoresis as described by Laemmli (1970). Fractions $E_{tot.}$, E_1 , E_2 , E_3 , E_4 and E_5 respectively are those described in Figs. 2 and 4.

Fig. 6. (a) Dioxygen dissociation curve of E_1 and (b) Hill plot of dioxygen equilibrium of E_1

(a) Measurements were performed in 50mM-Tris/HCl, pH 7.5, at 18°C, the diffusion-chamber technique of Sick & Gersonde (1969) being used; (b) data were calculated from (a). [Note: 1 mmHg $\equiv$ 133.3 Pa.] y is fractional saturation.

molecules by subtilisin (Moens *et al.*, 1984a), fraction E must contain a representative collection of the structural units present in the globin chains.

Analysis of this fraction by a two-dimensional technique (O'Farrell, 1975) reveals only three to four major spots, together with several minor ones (Fig. 3). Taking into account the aspecific cleavage by subtilisin, this strongly suggests the existence of only a limited number of different types of structural units. Spot E_A , (E_B) and E_C (M_r 15800) (Fig. 3) most likely represent the basal structural units of the globin chains.

As suggested by the cleavage kinetics (Moens *et al.*, 1984a), spot E_D seems rather to be a structural

Table 2. Amino acid composition of the structural unit E₁ and intact Hb II

Amino acid analysis of E₁ was performed as described in the Material and methods section. The values (% w/w) were the data obtained after 24h hydrolysis. Corrections were made as described by Reeck (1970).

Amino acid	Composition			
	Hb II*		E ₁	
	Residues/molecule of M _r 17000	(%, w/w)	(%, w/w)	Residues/molecule of M _r 17000
Asp	14.25	9.76	9.91	14.43
Thr	6.64	4.07	4.22	6.87
Ser	7.63	4.13	4.19	7.73
Glu	16.81	12.73	16.01	21.09
Phe	5.11	3.03	2.87	4.83
Gly	9.86	3.81	4.19	10.82
Ala	11.10	5.09	4.56	9.92
$\frac{1}{2}$ -Cys	1.11	0.69	—	—
Val	11.51	6.94	8.54	14.12
Met	2.65	2.04	0.86	1.12
Ile	7.66	5.17	3.60	5.32
Leu	15.75	10.63	11.99	17.72
Tyr	3.23	3.02	3.18	3.40
Phe	9.26	7.87	9.46	11.10
Trp	1.22	1.29	1.08	1.03
Lys	9.52	7.16	5.14	6.82
His	5.72	4.56	2.84	3.55
Arg	8.83	8.00	7.38	8.21
Total	148			148

* Moens & Kondo (1978).

unit(s) still connected with its linker region(s). An alternative interpretation is that it represents the N-terminal structural unit(s), which is (are) probably somewhat longer than the other ones.

The described method allows the purification to homogeneity of a single type of structural unit, E₁, that is still functionally intact. Milligram quantities can be obtained.

The method will allow us to determine the primary structure of E₁ (Moens *et al.*, 1984b; L. Moens, M.-L. Van Hauwaert, D. Geelen, G. Verpooten & J. Van Beeumen, unpublished work) and to compare it with the vertebrate globin sequences in order to reveal their structural and evolutionary relationship.

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