The globin of the nerve cord of the polychaete annelid Aphrodite aculeata was isolated and purified to homogeneity. The native molecule has a pI of 6.3 and acts as a dimer of two identical M, 15,644.5 polypeptide chains as determined by electrospray mass spectrometry. It has an average affinity for oxygen (P50 = 1.24 torr) resulting from fast association (k\text{on} = 170 \times 10^{6} M^{-1} s^{-1}) and dissociation rates (k\text{off} = 360 s^{-1}). The partial primary structure of this nerve globin was determined at the protein level and completed and confirmed by translation of the cDNA sequence. The globin chain has 150 amino acid residues and a calculated M, of 15,602.69 strongly suggesting that the amino terminus is acetylated. The absence of a leader sequence and the lack of Cys at the positions NA2 and H9 needed for the formation of the globin species. The Aphrodite nerve globin is unlikely to represent a separate globin family, as cDNA derived primers detect globin messenger RNA in muscle, gut, and pharynx tissue as well. The gene encoding this globin species is interrupted by a single intron, inserted at position G70. Comparison to other globin gene structures strongly suggest that introns can be lost independently, rather than simultaneously as a result of a single conversion event as suggested previously (Lewin, R. (1984) Science 226, 328).

Previously, no nerve globins have been characterized in detail. Therefore it is unclear whether they are an unique, novel globin type selectively expressed in nerve tissue or a normal "myoglobin" type also occurring in other tissues and over-expressed in nerve tissue.

In the ventral nerve cord of the marine polychaete Aphrodite aculeata, a monomeric Hb of M, 17,000 with a hyperbolic oxygen dissociation curve (P50 = 1.1 mm Hg) is present (2, 3). These characteristics are similar with those of the nerve globin of the mollusc Aplysia and strongly resemble these of a myoglobin type molecule.

Vertebrate and plant globin genes contain, respectively, two introns (in the B- and G-helix) and three introns (in the B-, E-, and G-helix). The intron insertion position is conserved at B12.2, E15.0, and G7.0. The three intron/four exon pattern of plants is proposed to be ancestral and all other globin gene structures would be derived mainly by intron loss (1). Several invertebrate and protozoan globin genes have been characterized and it has become clear that the intron/exon pattern is less conserved than originally expected (10, 11). For example, the intron/exon pattern of the globin gene of the annelid, Lumbricus terrestris, is the same as in vertebrates whereas that of some nematode globin genes is plant-like. However, at least five different insertion positions for the central (E-helix) intron are documented in nonvertebrates. Therefore the evolution of the intron pattern in the globin gene family has become a subject of debate (10–15).

Here we describe the kinetics of ligand binding of purified Aphrodite nerve globin, and the primary structure of the protein as determined by protein and cDNA sequence analysis. The structure of the gene encoding the globin polypeptide was determined as well.

MATERIALS AND METHODS

Purification of Aphrodite Nerve Globin—Live specimens of A. aculeata were collected in the North Sea or obtained from the University Biological Supply Millport, Scotland. The brilliant red ventral nerve
cord was dissected and frozen at −80°C until use. After thawing the nerve globin was allowed to diffuse from the tissue into 50 mM Tris-HCl, pH 7.5, 2 mM phenylmethylsulfonyl fluoride and concentrated by ammonium sulfate precipitation (3). The material precipitating between 40 and 75% ammonium sulfate was freed of minor contaminants by gel permeation chromatography on a Zorbax GF450 HPLC column according to previous published methods (16). Final purity was checked by one- and two-dimensional electrophoresis (17, 18).

Protein Sequencing—Heme extraction was performed by acid acetone precipitation. Globin samples were carboxymethylated, maleylated, and cleaved separately with trypsin and endoproteinase Asp-N (19). The resulting peptide mixtures were separated by RP-HPLC using a Vydac C4 column developed with 0.1% trifluoroacetic acid/CH$_3$CN. Peptides were sequenced in a ABI 471-B sequencer operated as recommended by the manufacturer. Globin sequence was partially reconstructed from relevant peptides using sperm whale myoglobin as a template (20, 21).

Mass Determination—Electrospray (ES) mass spectra were recorded on a VG Quattro II triple quadruple mass spectrometer (VG Manchester, UK) equipped with a Kontron HPLC system (Kontron Instruments). Tuning of the instrument was done by injecting a 20 pmol/μl solution of myoglobin. The electrospray carrier solvent used was CH$_3$CN/H$_2$O (50/50, v/v) containing 0.1% HCOOH. Samples were dissolved in the same solvent and injected via a Rheodyne loop injector of 100 μl. The flow rate of the carrier solvent was 40 μl/min. The capillary voltage was set at +3.81 kV. The source temperature was 80°C. The flow rate of the nebulizing gas and the drying gas were 200 l/h and 350 l/h, respectively. The cone voltage was 25 V. The mass spectrometer was scanned from mass to charge 500-1500. Spectra were recorded in the multichannel acquisition mode and by averaging 6 scans. The relative molecular mass ($M_r$) was calculated using Masslynx software.

Construction of Degenerate Oligonucleotide Primers—Two primers were designed based on the obtained protein sequence data. Primer APH1, CAYNGGNCAARTTYATGGA a 20-mer with 128 redundancies, corresponding to the sense strand predicted by the peptide fragment HGAKFME. Primer APH2, GTRTTRTANACYTTNGTCCA, also a 20-mer with 128 redundancies, corresponding to the antisense strand predicted by the peptide fragment TNYVKTW (Fig. 4).

cDNA Sequencing—mRNA was isolated from different tissues with a Fast Track mRNA isolation Kit (Invitrogen). Reverse transcriptase-PCR was carried out with the Stratagene kit. First strand cDNA was synthesized using an oligo(dT) primer. A PCR reaction was then performed using the degenerate primers. The PCR was carried out for 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min with Taq polymerase. The amplified fragment was blunt-end cloned into pBluescript KS$^+$, recombinants were confirmed by PCR, and the DNA sequence obtained from double stranded templates using T7 polymerase (Pharmacia Biotech Inc.) (22). Specific primers APH3 and APH4 were derived from the obtained DNA sequence (Fig. 5). APH3 and oligodT primer were used in a PCR reaction to obtain the 3' end of the cDNA. The 5' end was obtained with a 5' rapid amplification of cDNA ends experiment (RACE) (Life Technologies, Inc.) (23). First strand cDNA was synthesized with APH4 (specific antisense primer). A poly(C) tail was added to the 3' end of the cDNA with terminal deoxynucleotide

![Fig. 1. Analysis of Aphrodite nerve myoglobin fractions by 15% SDS-PAGE.](image1)

![Fig. 2. Absorption spectra of the Aphrodite nerve myoglobin.](image2)

![Fig. 3. Analysis of the dehemed and maleylated globin.](image3)

![Fig. 4. Reconstruction of the Aphrodite nerve myoglobin and comparison with the cDNA derived sequence.](image4)
transferase. The 5’ end was then amplified using an oligo(dG) adapter and the specific nested primer APH6 in a PCR of 30 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The sequence was determined as described earlier.

Genomic DNA—Isolated gDNA was used as a template in a PCR with primers APH3 and APH8 and in a PCR with primers APH5 and APH10. Fragments were cloned and sequenced as described earlier.

Binding Kinetics—Ligand rebinding kinetics were measured after photolysis with 10-ns laser pulses at 532 nm (Quantel, France). Samples were 10 μM in protein and equilibrated under 1 atm oxygen (1.2 mM at 20°C) or 1 atm CO (1 mM at 20°C). Detection of the kinetics was made at 436 nm, which is near the deoxy peak absorbance (Fig. 2).

For the CO dissociation rate, the ligand replacement method was employed. For oxygen displacement by CO, samples were prepared under a mixed CO/oxygen atmosphere. For nearly equal partial pressures of the two gases, the CO form is dominant, due to the higher affinity. After photodissociation, a large fraction of exposed hemes will bind oxygen since it has the higher on-rate. A second phase on a much slower time scale is the replacement of oxygen by CO to return to the original stable CO form. With the previously determined on-rates for oxygen and CO, one can simulate the kinetics to determine the oxygen off-rate.

For the CO dissociation rate, an aliquot of the CO sample was injected into a cuvette containing buffer with 1-5 mM potassium ferriyanide. The kinetics of the CO to Met transition were followed by measuring the absorption spectra every 3 s (initially) with a HP8453 diode array spectrophotometer.

Structural and Evolutional Analysis of Globin Sequence—The primary structure of Aphrodite nerve globin was aligned with relevant vertebrate and 145 nonvertebrate globins by means of existing templates (20, 21, 24). Penalty scores were calculated manually.

Based on this alignment, phylogenetic trees were constructed using the TREECON software (25).

RESULTS AND DISCUSSION

Isolation and Characterization of Nerve Hemoglobin—The nerve globin of Aphrodite was purified by ammonium sulfate precipitation and gel permeation chromatography as described under “Materials and Methods.” This results in a preparation essentially free of contaminants as judged by one-dimensional SDS-PAGE (Fig. 1).

The purified nerve globin shows absorption spectra in the oxy, deoxy, cyano-Met, and carbon monoxide forms essentially as reported previously (2). The spectrum of the deoxy derivative displays a shoulder at 550 nm, next to the maximum at 568 nm, confirming the earlier observations (2). Cytochrome b type spectral characteristics (maxima at 528 and 558 nm) as reported previously (2). The spectrum of the deoxy derivative displays a shoulder at 550 nm, next to the maximum at 568 nm, confirming the earlier observations (2). The apparent Mr of the native protein as determined by HPLC permeation chromatography was 31,600 ± 2,000. M, estimation by SDS-PAGE yielded 15,500 ± 400. After purification, extraction of the heme group, carboxymethylation, and maleylation, the nerve globin was subjected to RP-HPLC and a single globin chain was detected (Fig. 3A). Two-dimensional SDS-PAGE of this fraction revealed the presence of a single protein spot (Fig. 3B). Thus we conclude that the native protein is a homodimer. Similar conclusions were reached for the nerve globin of the bivalve species T. alternata and S. solidissima (3, 7). Interestingly Wittenberg et al. (2) calculated a Mr of 15,600 ± 1,000 for native Aphrodite globin from s20,w = 1.7. Concentration-dependent association/dissociation might be the cause of this discrepancy.

Determination of the Primary Structure—Amino-terminal sequencing of approximately 1 nmol of globin yielded no phosphorylation-derivative signal, suggesting that the polypeptide was inaccessible for Edman degradation. Sequence analysis of overlapping internal fragments permitted the construction of most of the polypeptide sequence as well as the construction of specific primers to amplify globin cDNA (Fig. 4). Full-length cDNA was isolated as described, and the sequence is presented in Fig. 5. It encompasses the entire coding region and confirms the amino acid sequence determined by Edman degradation. The initiation codon is preceded by at least 137 bases of untranslated sequence. No secretory leader sequence is present. This provides evidence that this globin is intracellular and a tissue or Mb type. The open reading frame extends for 150 codons and is followed by a 427-base long 3′-untranslated region ending with a polyadenylated tail. TATA box and polyadenylation signal are present.

| Species                  | Chain | Occurrence     | Accession     |
|-------------------------|-------|----------------|---------------|
| Echinodermata           | c, d  | Nucleated erythocytes | GLB-CAUAR, S15979 |
| Caulina arnica          |       | Nucleated erythocytes | S06134         |
| Paracaudina chilensis   |       | Nucleated erythocytes | GLB-CB, S15979 |
| Mollusca                |       | Nucleated erythocytes | S06503, GGNKT   |
| Anandara trapezia       | a, b  | Nucleated erythocytes | GLB-APLJU, S15979 |
| Aplysia limacina        |       | Radular muscle    | GLB-APLJU, S15979 |
| Aplysia juliana         |       | Radular muscle    | GLB-APLJU, S15979 |
| Cerithidea izophorarum  |       | Triturator stomach | A25331         |
| Dolabella auricularia   |       | Triturator stomach | A39023         |
| Bursatella leachii      |       | Triturator stomach | A39023         |
| Unicellulars            |       |                |               |
| Paramedium caudatum     | JQ1316|                |               |

Fig. 5. Sequence of the Aphrodite myoglobin gene derived from cDNA and gDNA. Primers are boxed and named. Partial intron sequence is given in lower case letters. TATA box and polyadenylation sites are marked in bold.

Table I: Acetylated amino termini in nonvertebrate globins
The M, calculated for the deduced polypeptide is 15,602.69. Using ESMS, a M, of 15,644.5 ± 0.48 was determined. Acetylation of the amino terminus would increase the mass by 43.03, which matches the difference of 41.81 ± 0.48 between measured and calculated M,. Hence, we conclude that the amino terminus is likely blocked by an acetyl group. Modified amino termini are not uncommon in globins in general. N-Acetylated termini are found in nonvertebrate globins intracellularly in erythrocytes or muscles but never in extracellular globins (Table I). The meaning of this difference is unclear.

The protein and cDNA derived sequence of the Aphrodite nerve globin is confirmed by the primary structure derived from the globin gene sequence (Fig. 5).

Structural and Phylogenetic Aspects of Duced Amino Acid Sequence—The alignment of the Aphrodite myoglobin sequence with the globin fold is unambiguous: (i) by the exclusion of polar residues from 33 out of the 33 invariant nonpolar sites (27), (ii) by the alignment of Pro-C2, which determines the folding of the BC corner, (iii) by the presence of the conserved heme-linked His (F8), and (iv) by the presence of the invariant Phel(CD1) (Fig. 6).

The Aphrodite nerve globin thus aligned matches both vertebrate and nonvertebrate globin templates quite well (20, 21). This is illustrated by the low penalty scores and proves that all major determinants of the globin fold are conserved (Table II).

The Phylogenetic tree of annelid and annelid related globin sequences was constructed using the neighbor-joining method (TREECON, 25). The bootstrap values are given at each branch point. The sequences used are: Phys (P. catodan); Glyc, Glycera dibranchiata globin MII; Tub, Tubifex tubifex globin I. In the heading of the alignment the major characteristics of the globin fold are summarized: helical notation (A to H), heme contacts (P. catodon: GLB1, GLB3, GLB5); Lum, Lumbricusterrestris; Tyl, Tylorrhynchus heterochaetus globin I; Lum, Lumbricusterrestris globin I; Tub, Tubifex tubifex globin I. In the heading of the alignment the major characteristics of the globin fold are summarized: helical notation (A to H), heme contacts (P. catodon: GLB1, GLB3, GLB5); Lum, Lumbricusterrestris; Tyl, Tylorrhynchus heterochaetus globin I; Lum, Lumbricusterrestris globin I; Tub, Tubifex tubifex globin I.

Fig. 6. Alignment of Aphrodite myoglobin with representative globin sequences. Mb, Glyc fold, myoglobin and Glycera fold, respectively; Phys, Phystester catodan; Glyc MII, Glycera dibranchiata globin MII; P, Aph, A. aculeata nerve globin; Tyl I, Tylorrhynchus heterochaetus globin I; Lum I, Lumbricusterrestris globin I; Tub I, Tubifex tubifex globin I. In the heading of the alignment the major characteristics of the globin fold are summarized: helical notation (A to H), heme contacts (p = proximal; d = distal), internal (lower case) and surface (capital) positions.

Table II

| A-motif | BC-motif | E-motif | F-motif | H-motif | Total |
|--------|----------|---------|---------|---------|-------|
| Phys   | NV V     | NV V    | NV V    | NV V    | NV V  |
| Glyc MII | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.2 0.5 |
| Glyc P1 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 |
| Tyl I  | 1.0 2.0 | 1.0 2.0 | 0.9 2.2 | 0.7 1.0 | 1.5 2.7 |
| Lum I  | 2.0 3.0 | 0.0 0.7 | 1.2 1.0 | 1.0 2.5 | 3.2 4.4 |
| Tub I  | 1.0 2.0 | 0.0 0.7 | 0.7 1.0 | 0.0 2.2 | 0.0 2.0 |
| Aphr   | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.2 0.6 | 0.0 0.0 |

Fig. 7. Phylogenetic tree of annelid and annelid related globin sequences. The abbreviations used are: Phys; P. catodan; Glyc, G. dibranchiata; Tyl, T. heterochaetus; Lum, L. terrestris; Tub, T. tubifex.

Fig. 8. Presence of globin mRNA in different tissues of Aphrodite. mRNA was prepared from gut (a), longitudinal muscle (b), nerve cord (c), and pharynx (d) tissue and used as template in a PCR reaction with APH3 and APH4 as primers. m, markers (BRL 1 Kb ladder).

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|--------|----------|---------|---------|---------|-------|
| Phys   | NV V     | NV V    | NV V    | NV V    | NV V  |
| Glyc MII | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.2 0.5 |
| Glyc P1 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 |
| Tyl I  | 1.0 2.0 | 1.0 2.0 | 0.9 2.2 | 0.7 1.0 | 1.5 2.7 |
| Lum I  | 2.0 3.0 | 0.0 0.7 | 1.2 1.0 | 1.0 2.5 | 3.2 4.4 |
| Tub I  | 1.0 2.0 | 0.0 0.7 | 0.7 1.0 | 0.0 2.2 | 0.0 2.0 |
| Aphr   | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 | 0.2 0.6 | 0.0 0.0 |

Fig. 8. Presence of globin mRNA in different tissues of Aphrodite. mRNA was prepared from gut (a), longitudinal muscle (b), nerve cord (c), and pharynx (d) tissue and used as template in a PCR reaction with APH3 and APH4 as primers. m, markers (BRL 1 Kb ladder).

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The Aphrodite nerve globin thus aligned matches both vertebrate and nonvertebrate globin templates quite well (20, 21). This is illustrated by the low penalty scores and proves that all major determinants of the globin fold are conserved (Table II).

Comparison with 145 nonvertebrate globin sequences reveals the highest similarity (31–32%) with the polymeric globins of the Glycera group (24, 28). All helical segments identified in sperm whale Mb and in the monomeric globin MII of Glycera (29) are also predicted to be present in the Aphrodite nerve globin. In addition, a 7-residue-long D-helix, typical of β type globin chains, is present. The distal heme ligand is provided by His as in most globins, including the polymeric Glycera globins. The presence of Leu at that position in Glycera globin MII in MIV is exceptional (28). Most heme contacts are conserved, although some are unusual. Lys(CD4) and Arg(F7) replace the common residues Tyr or Phe, respectively. Similar
**TABLE III**
Kinetic and equilibrium constants for the reactions of ferrous Aphrodite myoglobin with oxygen and carbon monoxide compared to those of other globins

| Protein          | $k_{on}$ | $k_{off}$ | $K_{assoc}$ ($k_{on}/k_{off}$) | $P50$ (torr) | $K_{diss}$ ($k_{off}/k_{on}$) | $l_{on}$ | $l_{off}$ | $L$ | Reference |
|------------------|----------|-----------|-------------------------------|-------------|-------------------------------|----------|----------|-----|-----------|
| Whale Mb         | 170      | 360       | 0.47                          | 1.24        | 2.1                           | 21       | 0.1      | 21  | This paper |
| Soybean Lb       | 24       | 130       | 0.18                          | 3.17        | 5.4                           | 0.98     | 0.012    | 82  | 37        |
| Glycera HbA      | 16       | 1.2       | 96.67                         | 0.006       | 0.01                          | 12.7     | 0.0078   | 1628| 38        |
| Glycera HbB      | 39       | 385       | 0.10                          | 5.8         | 9.9                           | 2.15     | 0.022    | 98  | 39        |
| Glycera HbC      | 186      | 1800      | 0.10                          | 5.7         | 9.7                           | 26.8     | 0.042    | 638 | 39        |
| Whale Mb         | 360      | 170       | 0.47                          | 1.24        | 2.1                           | 21       | 0.1      | 21  | This paper |
| Soybean Lb       | 130      | 24        | 0.18                          | 3.17        | 5.4                           | 0.98     | 0.012    | 82  | 37        |
| Glycera HbA      | 385      | 16        | 96.67                         | 0.006       | 0.01                          | 12.7     | 0.0078   | 1628| 38        |
| Glycera HbB      | 1800     | 39        | 0.10                          | 5.8         | 9.9                           | 2.15     | 0.022    | 98  | 39        |
| Glycera HbC      | 18       | 186       | 0.10                          | 5.7         | 9.7                           | 26.8     | 0.042    | 638 | 39        |

FIG. 9. Schematic representation of some representative globin genes. Open boxes are introns. Intron insertion is given by the helix notation of the amino acid codon that is interrupted. B12.2 indicates that the intron is inserted in the codon of the amino acid at position B12 after the second base. E15.0 indicates interruption between codons E14 and E15.

changes were also seen in the globin species present in the perienteric fluid in Ascaris, and as these positions coincide with surface crevices, it was suggested that both basic amino acids could form salt bridges with the heme propionates. The occupation of E11 by Phe is only shared by Lys(CD4) and Lys(E10) and might also form salt bridges with the polar groups of the heme propionates. It was suggested that both basic amino acids could force their polar groups to reach into the solvent (30).

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The structure of the Aphrodite globin gene is unusual. As mentioned before, globin genes mainly contain both the B and G helix introns together or neither of them. This intron configuration (B12.2 and G7.0) (Fig. 9) for example, occurs in the gene coding for the extracellular globin of the annelid, Lumbricus (12). The single G-helix intron in Aphrodite strongly suggests that the loss of individual introns can occur independently rather than simultaneously being caused by a single conversion event as suggested by Lewin (1). The presence of a single B-helix intron in the second domain of the Pseudoteranov globin gene confirms this view (35, 36).

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