Primary Structure of Two Linker Chains of the Extracellular Hemoglobin from the Polychaete Tylorrhynchus heteroachaetus*

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Two types of linker subunits (linkers 1 and 2) of the extracellular hemoglobin of Tylorrhynchus heteroachaetus have been isolated as disulfide-linked homodimers by C<sub>18</sub> reverse-phase chromatography. These subunits constituted 6 and 13%, respectively, of total protein area on the chromatogram. The complete amino acid sequences of linkers 1 and 2 were determined by automated Edman sequencing of the peptides derived by digestions with lysyl endopeptidase, trypsin, chymotrypsin, Staphylococcus aureus V8 protease, pepsin, and endoproteinase Asp-N. The linker 1 consisted of 253 amino acid residues (the calculated molecular mass, 28,200 Da), while the linker 2 consisted of 236 residues (28,316 Da). The two chains showed 27% sequence identity. The amino acid sequences of Tylorrhynchus linkers 1 and 2 also showed 23–27% homology with the recently determined sequence of a linker chain of Lamellibrachia hemoglobin (Suzuki, T., Takagi, T., and Ohta, S. (1990) J. Biol. Chem. 265, 1551–1555). In the three linker chains, half-cystine residues were highly conserved; 8 out of 13 residues are identical, suggesting that such residues would contribute to the formation of interchain disulfide bonds essential for the protein folding of the linker polypeptides. Based on the exact molecular masses of the linker and the heme-containing subunits, the molar ratios estimated for the subunits and the minimum molecular weights per 1 mol of heme, a model is proposed for the subunit structure of the Tylorrhynchus hemoglobin, consisting of 216 polypeptide chains, 192 heme-containing chains, and 24 linker chains.

Annelid extracellular hemoglobins are giant multia subunit proteins with molecular masses of 3000–4000 kDa. Most of the oligochaete and polychaete molecules are composed of two heme-containing subunits: a disulfide-bonded trimer of three different polypeptide chains (17–18 kDa) and a 16-kDa monomer (1); in addition, it was suggested that two or more 31–37-kDa chains which probably do not contain heme, act as “linkers” for the assembly of the heme-containing subunits in the case of Lumbricus and Lamellibrachia hemoglobins (2–4).

Direct structural evidence that the 31–37-kDa chains function as linkers has not been obtained as yet. However, the following results support this possibility. (i) A fraction of subunits deficient in the two 31–37-kDa chains of Lumbricus hemoglobin does not reassociate into whole molecule (5, 6). (ii) The deepsea tube worm Lamellibrachia contains two giant hemoglobins, a 3000-kDa hemoglobin and a 440-kDa hemoglobin. The former consists of four heme-containing chains and two 32–36-kDa linker chains, but the latter consists of only four heme-containing chains. Two of the four heme-containing chains are common to both hemoglobins and the other heme-containing chains are also homologous (4).

The complete amino acid sequence of one linker chain of Lamellibrachia 3000-kDa hemoglobin has been determined (7). The sequence suggested that the linker resulted from gene duplication of a heme-containing chain with a three exon–two intron structure, and that the first exon of domain 1 and the last exon of domain 2 had been lost during evolution (7).

In the present study, we succeeded in isolating two linker subunits of Tylorrhynchus hemoglobin, whose all four heme-containing chains have been already sequenced (8, 9), and determined the complete amino acid sequences of the two linker chains. The sequences were shown to have significant homology with that of the Lamellibrachia linker, especially with respect to the positions of half-cystine residues.

MATERIALS AND METHODS

About 300 g of worms that had been stored at −40 or −80 °C were homogenized with 900 ml of cold 50 mM phosphate buffer (pH 7.2) containing 0.5 mM phenylmethanesulfonyl fluoride and the supernatant was fractionated with 35–45% saturated ammonium sulfate. The material precipitated was dissolved in 250 ml of 50 mM phosphate buffer (pH 7.2) and was centrifuged at 50,000 rpm for 1 h. The hemoglobin fraction precipitated was dissolved in a minimum volume of the same buffer, applied to a Sepharose CL-4B column (3 × 110 cm) and eluted with the same buffer. The fractions of eluate containing hemoglobin were concentrated by ammonium sulfate precipitation, and applied again to the Sepharose CL-4B column. The purified hemoglobin was stored in a refrigerator at 2 °C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the absence or presence of 2-mercaptoethanol was performed as described previously (4).

The intact hemoglobin solution (0.3–0.6 mg) was applied directly to a reverse-phase column (Cosmosil 5C<sub>18</sub>-300, 4.6 × 150 mm, NaAlai Tesque) equilibrated with 30% acetonitrile in 0.1% trifluoroacetic acid, and eluted with a linear gradient of 30–60% acetonitrile, at a flow rate of 1 ml/min.

The two linker chains were carboxymethylated as described previously (8). The linker 1 (10 nmol) was digested with 10 μg of lysyl endopeptidase (Wako) in 50 mM Tris-HCl buffer (pH 8.8) at 37 °C for 3 h. The linker 1 (10 nmol) was also digested with 10 μg of trypsin ( Worthington) in 0.1 M ammonium bicarbonate at 37 °C for 1 h, and...
Asp-N (Boehringer Mannheim) in 50 mM Tris-HCl buffer (pH 8), with 10 μg of pepsin (Sigma) in 5% HCOOH at 37 °C for 0.5 h, and with 2 μg of endoproteinase Asp-N (Boehringer Mannheim) in 50 mM phosphate buffer (pH 8) at 33 °C for 21 h, respectively.

The linker 1 (14 nmol each) was digested with lysyl endopeptidase, chymotrypsin (in 0.1 M ammonium bicarbonate at 37 °C for 1.5 h), and endoproteinase Asp-N, respectively. The long peptides (L3 and L8) were digested further with chymotrypsin or trypsin.

The peptides were purified on a reverse-phase column (Cosmosil 5C18-300) with a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min. Some peptides were purified further by rechromatography.

Peptides were routinely hydrolyzed with HCl/trifluoroacetic acid (2:1, v/v) containing 0.02% phenol at 170 °C for 30 min in evacuated sealed tubes. Amino acid analysis was performed with a Hitachi 835-50 amino acid analyzer.

The amino acid sequences of the intact proteins and the isolated peptides were obtained using an automated Sequencer (Applied Biosystems model 477A).

The heme content of Tylorrhynchus hemoglobin was estimated from heme analysis (determination by the pyridine-hemochromogen method using a molar extinction coefficient of 32,000 at 557 nm) and amino acid analysis of the protein as described above. For the latter analysis, tryptophan was excluded from calculations.

**RESULTS**

Fig. 1 shows the SDS-PAGE patterns of Tylorrhynchus hemoglobin and of its subunits in the absence or presence of a reducing agent. The unreduced intact molecule dissociated into four subunits (lane 1): subunits L1 and L2 (55 kDa), subunit T (41 kDa), and subunit M (14 kDa). It is already known that subunit T is a disulfide-bonded trimer of three heme-containing chains and the subunit M is a monomeric chain (10). Upon reduction (lane 5), subunits L1 and L2 dissociated further into 34–36-kDa polypeptide chains, which are about double the size of usual heme-containing chains. Thus, subunits L1 and L2 appeared to be disulfide-bonded dimers. The molecular masses of the constituent polypeptide chains of subunits L1 and L2 were estimated to be about 28 kDa, from the mobilities of the unreduced subunits.

The subunits of Tylorrhynchus hemoglobin were separated on a reverse-phase column, as shown in Fig. 2. Four major fractions were eluted, but the third fraction with an absorbance at 220 nm (data not shown) was identified as heme. The recovery of the protein was more than 80%. The SDS-PAGE of fractions 1, 2, and 4 are shown in lanes 2–4 (unreduced) and lanes 6–8 (reduced) of Fig. 1. Fractions 1, 2, and 4 corresponded to subunits L1, L2, and T + M, respectively.

The heme and amino acid analyses showed that Tylorrhynchus hemoglobin contains 1 g eq of heme per 20,690 ± 1,460 g of protein (n = 5).

The amino acid sequence of Tylorrhynchus linker subunit 1 was determined as follows. Carboxymethylated protein was digested with lysyl endopeptidase (Fig. 3), trypsin (Figs. 4 and 5), S. aureus V8 protease (Fig. 6), pepsin (Fig. 7), and endoproteinase Asp-N (Fig. 8). Amino acid compositions and the results of amino acid sequencing of intact protein and its peptides are given in Tables I and II, respectively. The strategy used to establish the complete sequence is shown in Fig. 9.

The nature of the amino acid residue at position 7 of Tylorrhynchus linker 1 is uncertain. The amino acid sequence at positions 7 and 8 was determined to be Asp-Gly using peptide L1 (see Fig. 9), although yields of Edman cycles became much lower after position 7. We assumed that such low amino acid yields are derived from a cyclized Asn-Gly sequence, and that the original residue at position 7 is Asn and not Asp. The sequence proceeds beyond residue 7 due to a small amount of Asp generated during the isolation procedure of the peptides.

Tylorrhynchus linker chain 1 constituted of 253 amino acid residues, contained 12 half-cystines, and had a calculated molecular mass of 28,200 Da. The isoelectric point of linker 1 was calculated to be 6.2 from the exact amino acid composition.

The amino acid sequence of Tylorrhynchus linker 2 was determined by digestions with lysyl endopeptidase (Figs. 10–12), chymotrypsin (Fig. 13) and endoproteinase Asp-N (Fig. 14). Amino acid compositions and the results of amino acid sequencing of intact protein and its peptides are given in Tables III and IV, respectively. The strategy used to establish the complete sequence is shown in Fig. 15. Tylorrhynchus linker 2 consisted of 296 amino acid residues, contains 12

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Footnote: Portions of this work (including Figs. 3–15 and Tables I–IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
They form disulfide-bonded homodimers of ~28-kDa polypeptide chains. These subunits constituted 6 and 13% of total protein area on the chromatogram, respectively. In all cases investigated so far, the linkers show a more hydrophilic character relative to heme-containing subunits on reverse-phase chromatography. Linker chains would be non-heme proteins, since the minimum molecular mass per 1 mol of heme of Tylorrhynchus hemoglobin was estimated to be 20,680 g, a higher value when compared with those (16,000–17,000 g) of usual hemoglobins.

Gotob (12) and Suzuki et al. (10) could not find any major protein bands on SDS-PAGE corresponding to linkers in Tylorrhynchus hemoglobin. This indicates that the linkers were completely lost during hemoglobin preparation. One of the reasons might be due to an addition of 1 mM EDTA to the hemoglobin solution, which is known to protect hemoglobin from oxidation but also to chelate calcium and magnesium ions required for stability of the giant molecular architecture (5).

The SDS-PAGE patterns of Tylorrhynchus linker subunits in the absence or presence of a reducing agent showed that they form disulfide-bonded homodimers of ~28-kDa polypeptide chains. This is consistent with the results of the linkers of Arenocola (23), Nephys (13), Perinereis (14), and Neanthes (3) extracellular hemoglobins. But it is also reported that in many cases such as Lumbricus and Lamellibrachia hemoglobins (1, 4), the linkers do not form disulfide-bonded dimers. Even in the latter cases it is still possible that the monomeric linkers also form dimers in intact giant hemoglobins.

Amino acid sequence data are available for several linkers, including the N-terminal 28 residues of Lumbricus chain D1A (15), the complete sequence and N-terminal 17 residues of Lamellibrachia chains AV and AVI (7, 16), and the complete sequences of Tylorrhynchus linkers 1 and 2 (this work). Fig. 16 compares these N-terminal sequences. In our alignment, only three residues (Gln-44, Arg-47, and Leu-51) appear to be invariant, and the N-terminal sequences exhibit a remarkable variation. We believe that such a variation would not be due to a proteolytic cleavage, since a protease inhibitor was used in the isolation procedure of the hemoglobins. In any event, it is impossible at this stage to draw any conclusion about the evolutionary relationship between the sequences.

The complete amino acid sequences of Tylorrhynchus linkers 1 and 2 are aligned with that of Lamellibrachia linker (chain AV) (7) by a computer program based on the algorithm of Feng et al. (17), in Fig. 17. The sequence of Tylorrhynchus linker 1 shows 27 and 23% identity, respectively, with those of Tylorrhynchus linker 2 and Lamellibrachia linker (7). The sequence homology between Tylorrhynchus linker 2 and Lamellibrachia linker is 97%. In this alignment, 33 residues appear to be invariant, in which 8 half-cystine residues conserved exactly. Four half-cystine residues found only in Tylorrhynchus linkers 1 and 2 are especially noted. Such residues would contribute to the formation of some intrachain disulfide bonds essential for the stability of the linker chains. (5). The residues conserved in the three chains are shown by asterisks. Boxes indicate the positions of half-cystine residues.
Linker Chains of Tylorrhynchus Hemoglobin

TABLE V
Subunit structure for the 1/12th submultiple of Tylorrhynchus hemoglobin

| Subunit | Peak area from reverse-phase chromatography | Molecular weight of subunit | No. of copies | No. of chains | Contribution to molecular weight (Percent) |
|---------|-------------------------------------------|----------------------------|--------------|--------------|------------------------------------------|
| T + M   | 81                                        | 68.2*                      | 4            | (16)         | 273.0* (82)                              |
| L1       | 6                                         | 53.8'                      | 1            | (2)          | 53.8 (18)                                |
| L2       | 13                                        |                            |              |              |                                          |
| Total    | 100                                       |                            | 5            | (18)         | 326.8 kDa (100)                          |

*Calculated from amino acid sequences and heme groups.

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### Table 1: Amino acid compositions of intact protein and peptides of Tylorrhynchus Hemoglobin

| L1  | L2  | L3  | L4  | L5  | L6  | L7  |
|-----|-----|-----|-----|-----|-----|-----|
| 8.26 | 4.04 | 2.02 | 4.04 | 1.10 | 3.06 |
| Thr | 2.02 | 0.91 | 1.10 | 2.02 | 0.91 |
| Ser | 2.02 | 1.10 | 2.02 | 1.10 |
| Ala | 2.02 | 1.10 |
| Gly | 2.02 | 1.10 |
| Val | 2.02 | 1.10 |
| Ile | 2.02 | 1.10 |
| Leu | 2.02 | 1.10 |
| Asp | 2.02 | 1.10 |
| Glu | 2.02 | 1.10 |
| Phe | 2.02 | 1.10 |
| His | 2.02 | 1.10 |
| Tyr | 2.02 | 1.10 |
| Trp | 2.02 | 1.10 |
| Pro | 2.02 | 1.10 |
| Met | 2.02 | 1.10 |
| Cys | 2.02 | 1.10 |
| Total | 17 | 27 | 4 | 12 | 11 | 17 | 17 |
| Percent (%) | 57 | 71 | 13 | 37 | 33 | 57 | 57 |

### Table 1 (continued)

| L1  | L2  | L3  | L4  | L5  | L6  | L7  |
|-----|-----|-----|-----|-----|-----|-----|
| 8.26 | 4.04 | 2.02 | 4.04 | 1.10 | 3.06 |
| Thr | 2.02 | 0.91 | 1.10 | 2.02 | 0.91 |
| Ser | 2.02 | 1.10 | 2.02 | 1.10 |
| Ala | 2.02 | 1.10 |
| Gly | 2.02 | 1.10 |
| Val | 2.02 | 1.10 |
| Ile | 2.02 | 1.10 |
| Leu | 2.02 | 1.10 |
| Asp | 2.02 | 1.10 |
| Glu | 2.02 | 1.10 |
| Phe | 2.02 | 1.10 |
| His | 2.02 | 1.10 |
| Tyr | 2.02 | 1.10 |
| Trp | 2.02 | 1.10 |
| Pro | 2.02 | 1.10 |
| Met | 2.02 | 1.10 |
| Cys | 2.02 | 1.10 |
| Total | 17 | 27 | 4 | 12 | 11 | 17 | 17 |
| Percent (%) | 57 | 71 | 13 | 37 | 33 | 57 | 57 |

### Table 1 (continued)

| L1  | L2  | L3  | L4  | L5  | L6  | L7  |
|-----|-----|-----|-----|-----|-----|-----|
| 8.26 | 4.04 | 2.02 | 4.04 | 1.10 | 3.06 |
| Thr | 2.02 | 0.91 | 1.10 | 2.02 | 0.91 |
| Ser | 2.02 | 1.10 | 2.02 | 1.10 |
| Ala | 2.02 | 1.10 |
| Gly | 2.02 | 1.10 |
| Val | 2.02 | 1.10 |
| Ile | 2.02 | 1.10 |
| Leu | 2.02 | 1.10 |
| Asp | 2.02 | 1.10 |
| Glu | 2.02 | 1.10 |
| Phe | 2.02 | 1.10 |
| His | 2.02 | 1.10 |
| Tyr | 2.02 | 1.10 |
| Trp | 2.02 | 1.10 |
| Pro | 2.02 | 1.10 |
| Met | 2.02 | 1.10 |
| Cys | 2.02 | 1.10 |
| Total | 17 | 27 | 4 | 12 | 11 | 17 | 17 |
| Percent (%) | 57 | 71 | 13 | 37 | 33 | 57 | 57 |

### Table 1 (continued)

| L1  | L2  | L3  | L4  | L5  | L6  | L7  |
|-----|-----|-----|-----|-----|-----|-----|
| 8.26 | 4.04 | 2.02 | 4.04 | 1.10 | 3.06 |
| Thr | 2.02 | 0.91 | 1.10 | 2.02 | 0.91 |
| Ser | 2.02 | 1.10 | 2.02 | 1.10 |
| Ala | 2.02 | 1.10 |
| Gly | 2.02 | 1.10 |
| Val | 2.02 | 1.10 |
| Ile | 2.02 | 1.10 |
| Leu | 2.02 | 1.10 |
| Asp | 2.02 | 1.10 |
| Glu | 2.02 | 1.10 |
| Phe | 2.02 | 1.10 |
| His | 2.02 | 1.10 |
| Tyr | 2.02 | 1.10 |
| Trp | 2.02 | 1.10 |
| Pro | 2.02 | 1.10 |
| Met | 2.02 | 1.10 |
| Cys | 2.02 | 1.10 |
| Total | 17 | 27 | 4 | 12 | 11 | 17 | 17 |
| Percent (%) | 57 | 71 | 13 | 37 | 33 | 57 | 57 |
### Table II (continued)

| R  | L  | A  | N  | Q  | G  | H  | E  | V  | D  | N  | K  | T  | S  |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 40 | 10 | 15 | 11 | 9  | 0  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| 50 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |

### Table III (continued)

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table IV

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table V

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table VI

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table VII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table VIII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table IX

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table X

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XI

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XIII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XIV

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XV

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XVI

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XVII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XVIII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XIX

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XX

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXI

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXIII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXIV

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXV

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXVI

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXVII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXVIII

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXIX

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |

### Table XXX

| L  | L  | L  | C  | L  | L  | L  |
|----|----|----|----|----|----|----|
| 18 | 18 | 18 | 18 | 18 | 18 | 18 |
### Table III (continued)

| AS | A4 | AS-AS | CI | D2 | C3 | whole |
|----|----|-------|---|----|----|-------|
| 40 | 3.4(1) | 2.1(1) | 6.1(2) | 1.0(3) | 7.8(2) | 6.6(4) |
| 75 | 1.8(2) | 0.9(1) | 6.8(3) | 0.6(4) | 6.4(5) | 6.4(6) |
| 100 | 1.2(1) | 1.0(2) | 2.2(3) | 0.6(4) | 1.0(5) | 1.0(6) |
| 250 | 1.1(1) | 1.0(2) | 2.0(3) | 0.8(4) | 1.1(5) | 1.1(6) |
| 500 | 1.1(1) | 1.0(2) | 2.0(3) | 0.8(4) | 1.1(5) | 1.1(6) |
| 1000 | 1.1(1) | 1.0(2) | 2.0(3) | 0.8(4) | 1.1(5) | 1.1(6) |
| 2000 | 1.1(1) | 1.0(2) | 2.0(3) | 0.8(4) | 1.1(5) | 1.1(6) |

**Note:** Values are given in units of ng/mL. **Table IV (continued)**

| 100 | 2.0 | 1.0 |
| 200 | 2.0 | 1.0 |
| 300 | 2.0 | 1.0 |
| 400 | 2.0 | 1.0 |
| 500 | 2.0 | 1.0 |
| 600 | 2.0 | 1.0 |
| 700 | 2.0 | 1.0 |
| 800 | 2.0 | 1.0 |
| 900 | 2.0 | 1.0 |

**Note:** Values are given in units of ng/mL.
Linker Chains of Tylorrhynchus Hemoglobin

**Figure 1:** Reverse-phase chromatography of intact peptides of Tylorrhynchus hemoglobin. The column (Lichrosorb RP-18 25 cm) was equilibrated with 0.1% TFA, and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min.

**Figure 2:** Reverse-phase chromatography of post-gelatinase peptides of Tylorrhynchus hemoglobin. The column (Lichrosorb RP-18 25 cm) was equilibrated with 0.1% TFA, and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min.

**Figure 3:** Reverse-phase chromatography of post-gelatinase peptides of Tylorrhynchus hemoglobin. The column (Lichrosorb RP-18 25 cm) was equilibrated with 0.1% TFA, and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min.

**Figure 4:** Reverse-phase chromatography of post-gelatinase peptides of Tylorrhynchus hemoglobin. The column (Lichrosorb RP-18 25 cm) was equilibrated with 0.1% TFA, and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min.
Linker Chains of Tylorrhynchus Hemoglobin

Fig. 7. Reversed-phase chromatography of tryptic peptides of Tylorrhynchus hemoglobin, L. The elution gradient along with the eluate was equilibrated with 0.1% TFA and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 ml/min.

Fig. 8. Reversed-phase chromatography of endoproteinase Asp-N peptides of Tylorrhynchus hemoglobin. L. The elution gradient along with the eluate was equilibrated with 0.1% TFA and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 ml/min.

Fig. 9. Summary of data to establish the amino acid sequence of Tylorrhynchus hemoglobin. L. Amino acid sequence (1-10) was employed for sequence determination. Key: L, a tryptophan-containing peptide; T, a tryptic peptide; N, a non-tryptic peptide; B, a basic peptide; A, an N-terminal Asp-N peptide.

Fig. 10. Reversed-phase chromatography of endoproteinase carboxy peptides of Tylorrhynchus hemoglobin. L. The elution gradient along with the eluate was equilibrated with 0.1% TFA and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 ml/min.
Linker Chains of Tylorrhynchus Hemoglobin

Fig. 11. Reverse-phase chromatography of tryptic peptides of the peptide L3. The column (Shandon SGp-300) was equilibrated with 0.1% TFA and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 0.5 mL/min.

Fig. 14. Reverse-phase chromatography of chymotrypsin A-H peptides of Tylorrhynchus linker 2. The column (Shandon SGp-300) was equilibrated with 0.1% TFA and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 0.5 mL/min.

Fig. 12. Reverse-phase chromatography of tryptic peptides of the peptide L6. The column (Shandon SGp-300) was equilibrated with 0.1% TFA and eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 0.5 mL/min.

Fig. 15. Summary of data to explain the amino acid sequence of Tylorrhynchus linker 2. Amino acid sequence: 1, L2 was sequenced for sequence determination. Key: L, a link homology peptide; T, a tryptic peptide; C, a chymotrypsin peptide; A, a chymotrypsin A-H peptide.