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Studies on Monotreme Proteins. IV* Amino Acid Sequence of Haemoglobin-IA of the Echidna; A Comparison of Major Haemoglobins from Two Geographical Groups of Echidnas

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Abstract

The amino acid sequences of the α- and β-chains from a major haemoglobin (Hb-IA) of an echidna geographically isolated from those animals previously studied have been determined. The β-chain of Hb-IA was identical in amino acid sequence with the β-chain in Hb-IB. The α-chain of Hb-IA varied in four positions from that in Hb-IB, and had one more acidic group, in line with the higher electrophoretic mobility of Hb-IA at pH 8·5. There were no differences in 'contact site' residues in the α-chains of the two haemoglobins.

Introduction

Echidnas exhibit polymorphism in their haemoglobins. The different haemoglobins can be distinguished electrophoretically (Cooper *et al.* 1973) and three common variants of the major haemoglobin have been found, designated Hb-IA, Hb-IB and Hb-IC. Minor haemoglobins Hb-II with electrophoretic variants A and B are also present.

In previous papers (Whittaker *et al.* 1972, 1973) the fractionation of the haemoglobins from the south-eastern Australian echidna, *Tachyglossus aculeatus aculeatus*, was reported, together with the amino acid sequences of the α- and β-chains of the major haemoglobin Hb-IB. More recently (Thompson *et al.* 1973), the amino acid sequences of the α- and β-chains from the minor haemoglobin Hb-IIA, which is common to mainland animals, were determined. The β-chain was identical to that in Hb-IB whereas the α-chains varied in nine positions.

In the present communication the amino acid sequences of the α- and β-chains of the major haemoglobin Hb-IA in an animal from Western Australia are reported.

Materials and Methods

The haemoglobins Hb-IA and Hb-IIA were separated by chromatography on DEAE-Sephadex and the globins prepared as before (Whittaker *et al.* 1972). Fractionation of the α- and β-chains by chromatography on CM-cellulose in 8M urea–thiol buffers followed the method of Clegg *et al.* (1965) and the fractions were carboxymethylated before recovery of the protein. The methods of digestion with trypsin, chymotrypsin and thermolysin, fractionation of peptides and sequence determination by the dansyl–Edman procedure were the same as in previous papers (Whittaker *et al.* 1972, 1973).

Results

The separation of the α- and β-chains from the globin of Hb-IA was only partial and similar in this respect to the result previously reported for Hb-IIA (Thompson

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et al. 1973). As in that work, peptides from both α- and β-chains were fractionated by peptide-mapping techniques and assigned to the α- or β-chains from their position on the map and their similarity in amino acid sequence to known peptides from Hb-IB chains. Each β-globin tryptic peptide was sequenced by the dansyl–Edman method and the sequences were identical to those previously found in Hb-IB and Hb-IIA.

The α-chain peptides αTp1, αTp1+2A, αTp4, αTp5, αTp6A, αTp6B, αTp7, αTp8, αTp10, αTp11, αTp13A, αTp13B and αTp14 (see Table 1) were shown by the dansyl–Edman method and partial digestion with chymotrypsin or thermolysin to be identical in sequence to the same peptides from Hb-IB α-chain. The peptides with different amino acid sequences are discussed in turn in comparison with the corresponding peptides from the α-chain of Hb-IB (Whittaker *et al.* 1973).

αTp2B,3

Amino acid analysis showed an extra residue of glycine and no serine. The glycine was located at residue 4 in the peptide by dansyl–Edman degradation. Thermolysin digestion gave two peptides, Glu-Val-Thr-Gly and Leu-Trp-Gly-Lys, in confirmation of this arrangement.

αTp9

A sample of αTp9 from performic acid-oxidized globin that had been digested with trypsin was isolated from the largest size peptide fraction from a Sephadex G50 column, after further purification by peptide mapping. It was associated in the Sephadex column fraction with βTp10B+11,12A. Amino acid analysis showed one residue of serine less and one residue of aspartic acid or asparagine more than the corresponding Hb-IB peptide. Thermolysin digestion and sequence comparison of the peptides with those from a similar digest of Hb-IB α-chain showed that only the peptide Phe-Asx-Asx-Mes-Asx-Asx-Ala was different, with a serine residue being replaced by Asx (Mes = methionine sulphone). The mobility of this peptide at pH 6·4 suggested that there were two residues of aspartic acid and two of asparagine. The allocation of these amide groups was based on the ionophoretic mobility of peptides obtained by successively removing the *N*-terminal residue by the Edman method. A sample of each peptide was dansylated and their mobilities then determined at pH 6·4 relative to each other and to the sulphonic acid from the hydrolysis of the reagent (Gray 1972). The latter mobilities were:

Dns-Asx-Asx-Mes-Asx-Asx-Ala	1·4
Dns-Asx-Mes-Asx-Asx-Ala	1·5
Dns-Mes-Asx-Asx-Ala	1·1
Dns-Asx-Asx-Ala	1·2
Dns-Asx-Ala	0·6
Dns-Ala	0·8

which showed the sequence to be

Phe-Asn-Asp-Mes-Asp-Asn-Ala.

The distribution of amide groups on the first three Asx residues in this peptide was thus the same as in Hb-IB.

αTp12A

This peptide formed part of the insoluble 'core' remaining after tryptic digestion. After the globin had been labelled with [2-^{14}C]iodoacetate and repeatedly digested with trypsin at pH 8·7, the soluble peptides were removed and the insoluble residue was purified by gel filtration on Sephadex G50 in 10% formic acid. The radioactively labelled fraction was isolated and was clearly separated from βTp4, which can be detected from its high u.v. absorption due to tryptophan. Sequence determination by the dansyl–Edman method showed that both αTp12A and βTp12B were present in this fraction. Interpretation of the results, knowing the sequence of βTp12B, suggested the following sequence for αTp12A:

Leu-Leu-Ser-His-SCMCys-Phe-Leu-Val-Val-Leu-Ala-Arg,

with serine replacing the alanine residue at position 3 in the corresponding Hb-IB peptide.

Digestion of the 'core' fraction with chymotrypsin, followed by peptide mapping, gave a peptide with amino acid composition $His_{0.9}SCMCys_{0.8}Ser_1Leu_2Phe_1$, confirming the *N*-terminal sequence and the difference in sequence from Hb-IB. Digestion with thermolysin gave Leu-Ser-His-SCMCys, Leu-Gly-Asn (from βTp12B) and Ala-Arg, together with mixtures of neutral peptides containing phenylalanine, valine and leucine which were not purified. The sequences in these hydrophobic areas in both αTp12A and βTp12B are based on the dansyl–Edman degradations; the presence of dansylvalylvaline in hydrolysates at certain steps assisted in the interpretation of the data.

αTp12B

This peptide was in the same position on the peptide map as the variant αTp12B peptide in Hb-IIA, with a glutamic acid residue replacing the alanine at position 4 in the Hb-IB peptide. This sequence for the Hb-IA peptide was confirmed as in the earlier work (Whittaker *et al.* 1973; Thompson *et al.* 1973), by sequencing the fragments obtained after thermolysin digestion.

Complete Sequence

The complete sequence of the amino acid residues in the Hb-IA α-chain is shown in Table 1. The residues that are different in the α-chains of Hb-IB and Hb-IIA are shown above and below the variant residue respectively.

Discussion

Differences in the amino acid sequences of the α-chains of Hb-IA, Hb-IB and Hb-IIA give rise to differences in net negative charge at pH 8·5 which correspond to the differences in electrophoretic mobility of the three haemoglobins. Thus at pH 8·5 Hb-IIA, which moves most rapidly on electrophoresis (Cooper *et al.* 1973), has an α-chain with one negative charge more than Hb-IA α-chain and two negative charges more than Hb-IB α-chain. Since the β-chains are identical in sequence the tetrameric haemoglobins Hb-IA and Hb-IB must differ by two and four net negative charges respectively from Hb-IIA.

There are only four differences in amino acid sequence between Hb-IA and Hb-IB, while the Hb-IIA sequence shows nine differences from Hb-IB and ten differences from Hb-IA. All of the changes between Hb-IB and Hb-IA or Hb-IIA can be explained by single base changes in the mRNA. Between Hb-IA and Hb-IIA, the difference in one residue (78), which is asparagine and glycine respectively, would necessitate a minimum of two base changes.

Table 1. Amino acid sequence of the α-chain of echidna haemoglobin-IA

Residues are numbered from the *N*-terminal residue and the positions of cleavage by trypsin are indicated by arrows. Dotted arrows indicate positions of incomplete cleavage. The residues that differ in the Hb-IB sequence are shown above the variant position and those that differ in Hb-IIA are shown below

5 10 Ser 15 20
Val-Leu-Thr-Asp-Ala-Glu-Lys-Lys-Glu-Val-Thr-Gly-Leu-Trp-Gly-Lys-Ala-Ser-Gly-His-Ala-Glu-
Arg↑ ↑ Ser ↑
αTp1 αTp2A αTp2B,3

25 30 35 40
Glu-Tyr-Gly-Ala-Glu-Ala-Leu-Glu-Arg-Leu-Phe-Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Ser-
Asp ↑ ↑
αTp4 αTp5

45 50 55 60 65
His-Met-Asp-Leu-Ser-Lys-Gly-Ser-Ala-Gln-Val-Lys-Ala-His-Gly-Lys-Arg-Val-Ala-Asp-Ala-Leu-
↑ His Arg↑ ↑Lys↑
αTp6A αTp6B αTp7 αTp8

70 75 Ser 80 85
Thr-Thr-Ala-Ala-Gly-His-Phe-Asn-Asp-Met-Asp-Asn-Ala-Leu-Ser-Ala-Leu-Ser-Asp-Leu-His-Ala-
Val Gly Asp
αTp9

90 95 100 Ala 105 110
His-Lys-Leu-Arg-Val-Asp-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-Cys-Phe-Leu-Val-Val-Leu-Ala-
↑ ↑ ↑ Ala
αTp10 αTp11 αTp12A

Ala 120 125 130
Arg-His-His-Pro-Glu-Glu-Phe-Thr-Pro-Ser-Ala-His-Ala-Ala-Met-Asp-Lys-Phe-Leu-Ser-Arg-Val-
↑ ↑ ↑
αTp12B αTp13A

135 140
Ala-Thr-Val-Leu-Thr-Ser-Lys-Tyr-Arg
↑
αTp13B αTp14

Only blood from a single animal from Western Australia was available. The changes in amino acid sequence between the haemoglobin of this animal and those from south-eastern Australian animals previously investigated have not changed any of the 'contact sites' between the α- and β-chains (Perutz *et al.* 1968). There is at present no evidence to suggest whether the changes in amino acid sequence recorded for these polymorphic haemoglobins offer any functional advantage to the animals.

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