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Studies on Yak Hemoglobin (*Bos grunniens*, Bovidae): Structural Basis for High Intrinsic Oxygen Affinity?

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Summary: Two types of α- and two types of β-chains are found in the hemoglobin of yak population. The complete amino-acid sequences of the four polypeptide chains were determined. The two α-chains differ by two and the two β-chains by three amino-acid substitutions. The substitution of valine at position 135 in the β^{II}-chain may be responsible for the high intrinsic oxygen affinity of yak hemoglobin.

Untersuchung der Hämoglobine des Yak (Bos grunniens, Bovidae): Eine strukturelle Basis für hohe intrinsische Sauerstoffaffinität?

Zusammenfassung: In der Yak-Population werden zwei Typen von α- und zwei Typen von β-Ketten der Hämoglobine gefunden. Es wurde die komplette Aminosäuresequenz von vier Peptidketten bestimmt. Die beiden α-Ketten unterscheiden sich nur in zwei Aminosäureresten, während die beiden β-Ketten drei Unterschiede zeigen. Die Substitution von Valin in Pos. 135 der β^{II}-Ketten könnte verantwortlich sein für die hohe intrinsische Sauerstoffaffinität der Yak-Hämoglobine.

Key words: Yak hemoglobin, amino-acid sequence, multiple substitution, intrinsic oxygen affinity, Bovidae.

Multiple forms of hemoglobins within species are common in the family Bovidae. The multiple hemoglobins are the result of differences in the primary structure of the α- or β-chains which are controlled by either allelic or non-allelic structural genes. Allelism is found in the hemoglobin β-chain locus of cattle[2] and in the α-chain locus of gayal[3] while non-allelic structural genes are found in the α-chain locus of water buffalo[4]. In goat population different hemoglobins produced by allelic α- and β-chain structural genes as well as non-allelic α-chain structural genes are found[5,6] while allelism is found in the β-chain locus of sheep[7]. Unlike in man where most of the variant hemoglobins are due to single amino-acid replacements, the different hemoglobin chains in these bovid populations have multiple site amino-acid substitutions. Moreover, the β-chains of these hemoglobins have one amino-acid deletion in the amino-terminal segment and the hemoglobins have low intrinsic oxygen affinity which is not much affected by 2,3-bisphos-

Abbreviations:
Quadrol = *N*,*N*,*N*′,*N*′-Tetrakis(2-hydroxypropyl)ethylenediamine;
TosPheCH$_2$Cl = (*N*-tosyl-L-phenylalanyl)chloromethane;
CM-Cellulose = carboxymethyl cellulose;
DEAE-Cellulose = diethylaminoethyl cellulose;
TpA = tryptic peptide obtained after blocking the ε-amino group of lysine; Tp = tryptic peptide; CNBr = cyanogen bromide peptide; AP = peptide obtained by acidic cleavage of Asp-Pro peptide bond.

* 77th Communication on hemoglobins; for 76th communication see ref.[1].

phoglycerate[8]. The yak *(Bos grunniens)*, which also belongs to the family Bovidae, is a high altitude animal and is well adapted to the low oxygen partial pressure prevailing in the high mountains of the Himalayas. The yak has been reported to have hemoglobin with higher oxygen affinity than its lowland relatives[9]. We are interested to find out whether there is any primary structural basis for the reported high oxygen affinity of yak hemoglobin. Moreover, when the present work was started, no complete sequence of the hemoglobins of Bovidae except that of the bovine fetal chain[10] was available. (In the meantime the complete sequences of the fetal chains of sheep and goat have been published[11]). Hence information on the complete amino-acid sequence is desirable for an understanding of the evolutionary relationships of the different species of Bovidae. With these objectives we have determined the amino-acid sequences of the α- and β-chains of yak hemoglobin[12,13]. In this paper, we give the experimental details and discuss the probable structural basis for the high intrinsic oxygen affinity of these hemoglobins.

Materials and Methods

Blood samples were obtained by vene puncture from three adult yaks and one month-old calf from Hellabrunn Zoo, München, using heparin as anticoagulant. The erythrocytes were separated from the plasma by centrifugation and washed three times with isotonic saline solution. The erythrocytes were lysed by adding an equal volume of distilled water and 0.4 volume toluene and the cell debris removed by centrifugation.

Hemoglobin was checked for heterogeneity by polyacrylamide disc gel electrophoresis[14] at pH 8.6. The number of different subunits present was determined by the Triton-polyacrylamide gel electrophoresis method of Alters et al.[15] as modified by Mezel et al.[16].

Globin was prepared by the acid-acetone precipitation method of Rossi-Fanelli and Antonini[17]. The different polypeptide chains were isolated by ion exchange chromatography on CM-cellulose (Whatman CM-52) according to Clegg et al.[18] and desalted by gel filtration. The sequences were determined on the intact polypeptide chains as well as their peptides.

Fragments were obtained by *chemical cleavages*[19,20] and *tryptic digestion* of the *S*-carboxymethylated[21] chains as well as of the *S*-carboxymethylated chains whose lysine ε-amino groups had been blocked with maleic anhydride[22]. Tryptic digestion using TosPheCH$_2$Cl-treated trypsin (Worthington) was carried out for 2 h at 38 °C at an enzyme-protein ratio of 1 : 50 (w/w). The peptides were fractionated by gel filtration on Sephadex G-50 with 8M urea/10% formic acid or 6M guanidinium chloride/10% formic acid. The tryptic peptides were fractionated on Sephadex G-25 (fine) using 0.1M acetic acid. Where necessary, the peptides were further purified by ion exchange chromatography.

Amino-acid analysis of the peptides was done in a Beckman amino-acid analyser model 121C after hydrolysis in 5.7M HCl at 110 °C for 20 h. Tryptophan was determined using 6% thioglycolic acid in 5.7M HCl.

The *amino-acid sequence* was obtained in a Beckman sequencer by automatic Edman degradation using Quadrol[23] and *N,N'*-diethylaminopropyne[24] programs. The amino-acids were identified as their phenylthiohydantoin derivatives by thin-layer chromatography on silica plates (Merck, Darmstadt) using the solvent system of Braunitzer et al.[25]. Whereever necessary, they were also identified by high performance liquid chromatography.

Results

On polyacrylamide gel electrophoresis at pH 8.6, the hemolysates from two adult yaks resolved into two bands and from one adult into three bands while the hemolysate from the calf resolved into four bands as shown in Fig. 1a. The hemolysates which showed two and three bands resolved into three and four bands respectively in Triton-polyacrylamide gel electrophoresis and the hemolysate from the calf resolved into four bands, three bands corresponding to the bands of adult hemolysates and one band different from the adult as shown in Fig. 1b. For the sequence determination the adult sample showing four different subunits was taken.

When the globin was chromatographed on CM-cellulose four elution peaks were obtained. The polypeptide chains were designated as β^{I}, β^{II}, α^{I}, α^{II} in the order of their elution from the CM-cellulose column. When the isolated chains were electrophoresed on Triton-polyacrylamide gel, it was found that the order of elution from the column was just the reverse of their cathodic mobility i.e. the α^{II} chain has the highest cathodic mobility and the β^{I} chain the lowest cathodic mobility. The hemolysate from calf was found to have two α-chains identical to the two α-chains of adult hemoglobin, one β-chain identical to β^{II} and one chain which was different from the adult hemoglobin chains and this was designated as γ-chain.

Cyanogen bromide cleavage of the chains resulted in two peptides in each of the α-chains while four peptides were obtained from each of the β-chains. By acidic cleavage of the Asp-Pro peptide bond, two peptides were obtained from each of the four chains. Tryptic digestion of the β-chains after blocking of the ε-groups resulted in five peptides from each of the β-chains.

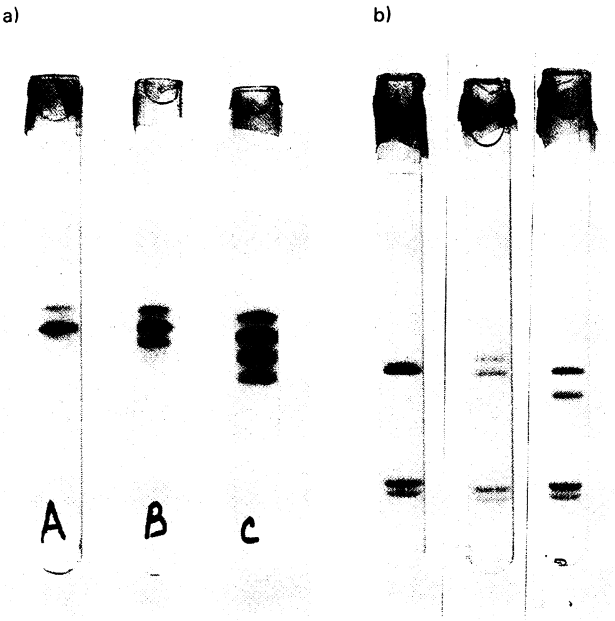

Fig. 1. Polyacrylamide gel electropherograms a) of yak hemoglobins and b) of the polypeptide chains in the presence of
A. Adult Hb homozygous for β^{II}.
B. Adult Hb heterozygous for β^{I} and β^{II}.
C. Fetal Hb + adult Hb homozygous for β^{II}.

The α-chain sequence was determined on the intact chain, CNBr 2, Tp 9, Tp 10, the C-terminal and penultimate residues of AP 1 (by digestion with carboxypeptidase A) and AP 2, whereas the β-chain sequence was determined on the intact chain, TpA 3, CNBr 4, AP 2, TpA4 and TpA 5. All these peptides except Tp 9, Tp 10 and TpA 4 were obtained pure by gel filtration on Sephadex G-50. After gel-filtration TpA 4 was further purified by ion exchange chromatography on DEAE-cellulose using 0.05M Tris/HCl buffer, pH 8.5 in the presence of 8M urea. αTp 9 and αTp 10 were isolated by gel filtration on Sephadex G 25 (fine) using 0.1M acetic acid as eluent.

The amino-acid composition of the α^{I}- and α^{II}-chains and their peptides is given in Table 1 and the amino-acid composition of the β^{I}- and β^{II}-chains and their peptides in Table 2 (Table 1 and 2, see Suppl. Material). The amino-acid sequence of the α- and β-chains are presented in Fig. 2.

Discussion

Our results and an earlier report on yak hemoglobin[26] showed that each individual animal in the population of yak had a minimum of two hemoglobin components while some animals have more than two. Each animal investigated had two types of α-chains. Some animals had only one type of β-chain while one had two types of β-chains. Thus there are two types of α-chains and two types of β-chains in the yak population. Consequently there are four types of hemoglobins, viz., $\alpha^{I}_2\beta^{I}_2$, $\alpha^{I}_2\beta^{II}_2$, $\alpha^{II}_2\beta^{I}_2$ and $\alpha^{II}_2\beta^{II}_2$. The four hemoglobin phenotypes are designated as Hb 1 ($\alpha^{I}_2\beta^{I}_2$), Hb 2 ($\alpha^{I}_2\beta^{II}_2$), Hb 3 ($\alpha^{II}_2\beta^{I}_2$) and Hb 4 ($\alpha^{II}_2\beta^{II}_2$). The two α-chains are products of non-allelic α-chain structural genes whereas, the two β-chains derive from allelic β-chain genes.

Animals homozygous for either of the β-chains will have two types of hemoglobins: $\alpha^{I}_2\beta^{I}_2$ and $\alpha^{II}_2\beta^{I}_2$ or $\alpha^{I}_2\beta^{II}_2$ and $\alpha^{II}_2\beta^{II}_2$; animals heterozygous for both the β-chains exhibit all the four hemoglobin types. Of the four animals investigated, three had β^{II} and only one had both β^{I}- and β^{II}-chains. Adam et al.[26] found only one type of β-chain in the hemoglobin from five yaks. Probably the frequency of occurence of the β^{II}-chain is much higher than that of the β^{I}-chain in the yak population.

The two α-chains differ at two residues, at position 50 and 71, with glutamine at position 50 and glutamic acid at position 71 in the α^{I}-chain. The corresponding amino acids in the α^{II}-chain are histidine and glycine, respectively. The two β-chains differ at three positions, namely at 50, 117 and 135, occupied by threonine, asparagine and alanine in β^{I}; and by serine, histidine and valine in β^{II}.

When the sequences of the α-chains are compared with that of bovine α-chain[27], bovine α-chain differs from each of the yak α-chains[12] by one amino-acid residue. The difference between bovine α and yak α^{I} is found at position 50 ($His^{bov.} \rightarrow Gln^{yak}$) and that between bovine α and yak α^{II} at position 71 ($Glu^{bov.} \rightarrow Gly^{yak}$). Bovine β^{A}[2,28] differs from yak β^{I} and β^{II}[13] by one and four residues, respectively: Bovine β^{A} and yak β^{I} show an Asp E17(73) $\beta^{A} \rightarrow$ Asn β^{I} substitution and the differences between bovine β^{A} and yak β^{II} are:

Thr D1 (50) $\beta^{A} \rightarrow$ Ser β^{II},
Asp E17 (73) $\beta^{A} \rightarrow$ Asn β^{II},
Asn G19 (117) $\beta^{A} \rightarrow$ His β^{II} and Ala H13 (135) $\beta^{A} \rightarrow$ Val β^{II}.

The most significant difference between hemoglobins with low intrinsic affinity for oxygen and those with high intrinsic affinity is the replacement of hydrophilic residues at NA2β in hemoglobins with high oxygen affinity by hydrophobic residues in hemoglobins with low

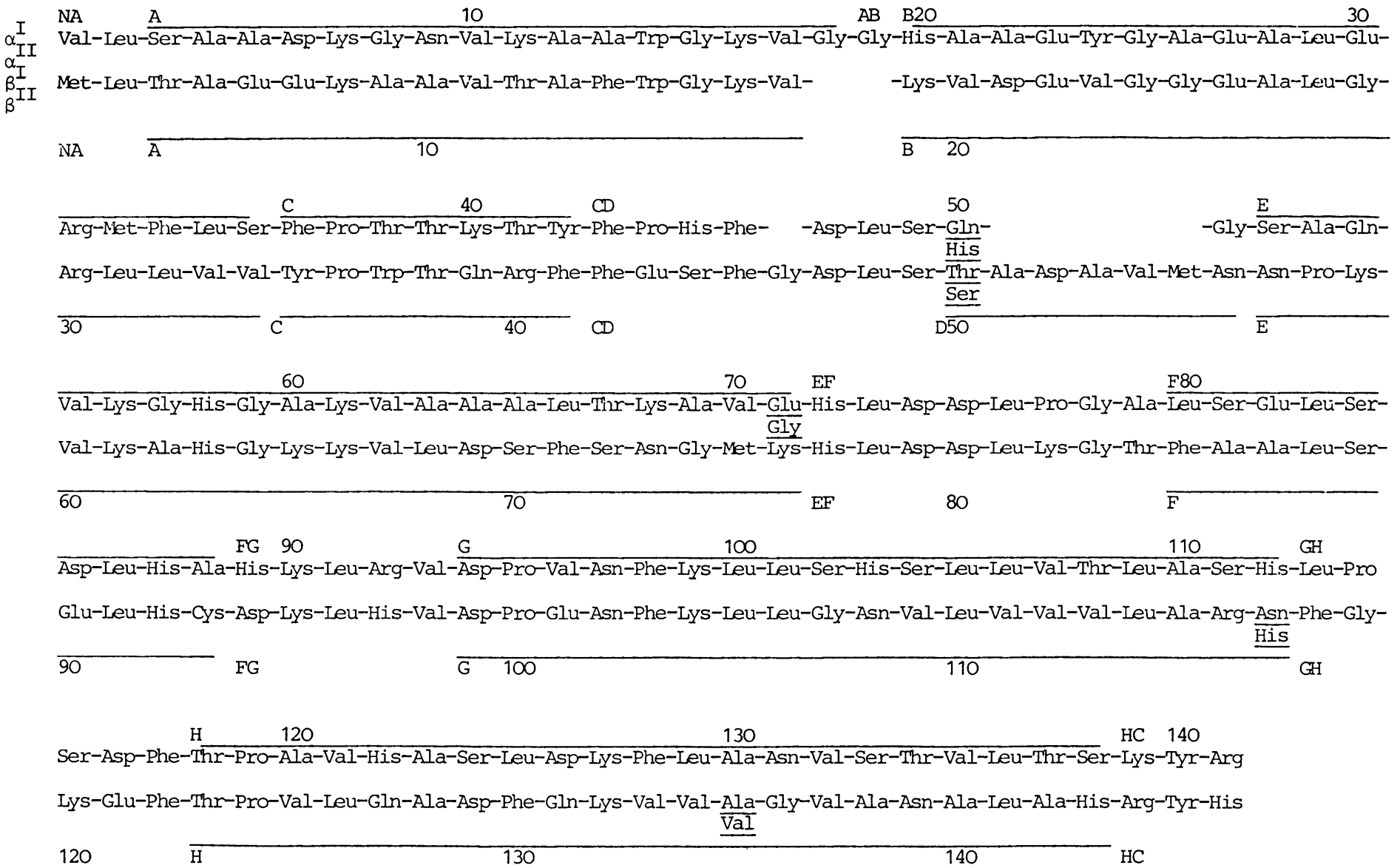

Fig. 2. Amino-acid sequences of yak hemoglobin α^{I}, α^{II}, β^{I} and β^{II} chains.

The sequences of the α^{II} and the β^{II} chains are indicated only at those positions where they differ from those of the α^{I} and the β^{I} chains respectively. One residue at Pos. 2 is deleted in the β-chain of ruminants. A residue number has been given to this deletion for homologous comparison with other β-chains.

oxygen affinity[29]. The β-chains of yak hemoglobin also have the same hydrophobic residue (methionine) at this position like those of cattle, sheep and goat. Yet yak hemoglobin has a higher intrinsic affinity for oxygen than the hemoglobins of its lowland relatives[9]. Most of the above-mentioned amino-acid replacements between bovine and yak β-chains are unlikely to cause changes in the oxygen affinity. However, yak β^{II} is unique in having valine at position 135. In the β-chain of all mammals except Echidna[30], in the fetal chains of cattle[10], sheep and goat[11] and in the γ-chain of human hemoglobin[31], alanine is found at this position. Although this alanine does not make direct contact with the heme, it is in the vicinity of the heme. The replacement by the larger side chain of valine might cause hydrophobic interaction in the heme environment and this may be responsible for the higher oxygen affinity of yak hemoglobin as compared to the hemoglobin of its lowland relatives. In Hb Altdorf this alanine is replaced by proline and the substitution disrupts the helix and causes higher oxygen affinity of this mutant hemoglobin[32]. Though we found two types of β-chains in the limited yak population we investigated, no animal was found to be homozygous for the β^{I}-chain. Perhaps the advantage of having a β^{II}-chain with higher oxygen affinity for the natural habitat of yak has favoured the selection of β^{II}-chain genes rather than β^{I}-chain genes, though it is premature to draw any conclusions at the moment.

Although the amino-acid substitutions in the two α-chains appear to be conservative, they seem to have some effect on the oxygen affinity. Yak Hb 2 and Hb 4, which have the same β^{II}-chain, show slight differences in their oxygen affinity (our unpublished data). Hb 2 with α^{I} has a higher affinity for oxygen than Hb 4 with α^{II}. Probably glutamine at position 50 in the α^{I}-chain is responsible for this increased affinity although the mechanism responsible is still unclear.

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Supplementary Material

Table 1. Amino-acid composition of α^{I} and α^{II}-chains and their peptides.

Pos.	Chain 1-141 α^{I}	Chain 1-141 α^{II}	CNBr 2 33-141 α^{I}	CNBr 2 33-141 α^{II}	Tp 9 69-90 α^{I}	Tp 9 69-90 α^{II}	Ap 2 95-141 α^{I}	Ap 2 95-141 α^{II}
Asp	11.2 (11)	11.3 (11)	9.0 (9)	9.3 (9)	3.2	2.9	4.1	4.2
Thr	7.8 (8)	7.7 (8)	7.6 (8)	7.8 (8)			3.8	3.8
Ser	12.7 (13)	12.6 (13)	11.6 (12)	11.7 (12)	2.1	2.0	6.8	6.9
Glu	7.1 (7)	5.2 (5)	4.6 (4)	2.7 (2)	2.1	1.1		
Pro	6.1 (6)	6.3 (6)	6.1 (6)	6.0 (6)	0.9	1.0	2.8	2.7
Gly	9.2 (9)	10.1 (10)	4.2 (4)	5.1 (5)	1.0	1.9		
Ala	20.3 (20)	20.2 (20)	12.1 (12)	11.9 (12)	3.3	2.9	4.2	4.1
Val	11.8 (12)	11.9 (12)	9.1 (9)	8.8 (9)	1.2	1.1	4.8	4.9
Met	0.8 (1)	0.8 (1)						
Leu	20.4 (20)	20.2 (20)	18.2 (18)	17.8 (18)	5.2	4.7	9.2	9.1
Tyr	2.8 (3)	2.7 (3)	2.1 (2)	1.9 (2)			0.8	0.9
Phe	7.1 (7)	7.2 (7)	6.8 (7)	6.9 (7)			3.2	3.3
Trp	0.8 (1)	0.9 (1)						
His	9.2 (9)	10.1 (10)	7.5 (8)	8.4 (9)	2.8	2.9	3.1	3.1
Lys	11.1 (11)	10.8 (11)	7.8 (8)	8.2 (8)	0.9	1.1	3.0	3.1
Arg	2.8 (3)	2.9 (3)	1.8 (2)	2.0 (2)			0.9	1.0
Total	141	141	109	109	22	22	47	47

Table 2. Amino-acid composition of β^{I} and β^{II}-chains and their peptides.

Pos.	Chain 1-146 β^{I}	Chain 1-146 β^{II}	TpA 3 41-116 β^{I}	TpA 3 41-116 β^{II}	CNBr 4 76-146 β^{I}	CNBr 4 76-146 β^{II}	AP 2 100-146 β^{I}	AP 2 100-146 β^{II}	TpA 4 117-144 β^{I}	TpA 4 117-144 β^{II}	TpA 5 145-146 β^{I}	TpA 5 145-146 β^{II}
Asp	16.1 (16)	15.3 (15)	12.1 (12)	12.3 (12)	9.3 (9)	8.1 (8)	5.2	4.0	3.0	2.1		
Thr	5.9 (6)	5.0 (5)	2.2 (2)	1.3 (1)	1.9 (2)	2.1 (2)	1.0	1.0	0.9	0.8		
Ser	4.7 (5)	5.8 (6)	5.1 (5)	5.8 (6)	0.9 (1)	0.8 (1)						
Glu	11.3 (11)	11.1 (11)	3.4 (3)	3.2 (3)	5.2 (5)	5.1 (5)	4.1	4.3	3.3	2.8		
Pro	3.8 (4)	3.7 (4)	1.9 (2)	1.9 (2)	1.9 (2)	2.0 (2)	1.8	1.7	0.9	0.8		
Gly	11.2 (11)	11.0 (11)	5.4 (5)	5.1 (5)	4.1 (4)	4.3 (4)	3.2	3.4	2.2	1.9		
Ala	16.3 (16)	15.2 (15)	6.3 (6)	6.1 (6)	8.0 (8)	7.1 (7)	6.2	5.0	5.2	4.0		
Val	17.8 (18)	18.7 (19)	7.8 (8)	7.7 (8)	8.6 (9)	9.8 (10)	7.9	8.8	3.8	5.1		
Met	2.7 (3)	2.6 (3)	1.8 (2)	1.7 (2)								
Leu	16.9 (17)	17.1 (17)	10.6 (11)	10.8 (11)	10.9 (11)	10.8 (11)	5.9	6.1	2.1	1.8		
Tyr	1.7 (2)	1.8 (2)			0.8 (1)	0.9 (1)	0.9	0.7			0.9	1.0
Phe	10.1 (10)	10.2 (10)	5.8 (6)	5.7 (6)	5.1 (5)	5.1 (5)	3.7	3.9	2.8	2.7		
Trp	1.8 (2)	1.7 (2)										
His	5.9 (6)	7.1 (7)	4.0 (4)	3.9 (4)	4.9 (5)	5.8 (6)	1.8	3.2	0.9	2.1	1.2	1.1
Lys	13.2 (13)	13.0 (13)	8.1 (8)	7.9 (8)	5.8 (6)	6.2 (6)	3.3	2.9	1.8	2.3		
Arg	4.1 (4)	4.2 (4)	1.1 (1)	1.2 (1)	1.8 (2)	2.0 (2)	2.1	1.9	0.9	0.9		
Cys	1.0 (1)	1.1 (1)	(1.0) (1)	(1.0) (1)	(1.0) (1)	(1.0) (1)						
Total	145	145	76	76	71	71	47	47	28	28	2	2