Characterization of hemoglobin from the lizard *Uromastix hardwickii*

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Hemoglobin from the tropic lizard *Uromastix hardwickii* was isolated. Chain separations were studied, and the whole carboxymethylated globin was cleaved with trypsin. Peptides were pre-fractionated by exclusion chromatography and finally purified by reversed phase high-performance liquid chromatography. Amino acid sequence analysis permitted ordering of peptides in α- and β-chains by homology with known structures in other hemoglobins. Results show large structural variations (about 50% homology between *Uromastix* and viper α-chains) and suggest chain heterogeneity with the presence of at least two types of both the α- and β-chains in the preparations.

Hemoglobin heterogeneity *Amino acid sequence analysis* *Homology*

1. INTRODUCTION

Hemoglobin is an evolutionarily well characterized protein, studied in many vertebrate species [1–3]. It exhibits comparatively rapid evolutionary changes [1,4] and few reptilian species have been analyzed. Comparison of viper hemoglobin α-chain [2] with mammalian, chicken and carp α-chains, shows 46 out of 141 residues to be invariant [1]. In relation to the human α-chain, the viper substitutions are unevenly distributed along the polypeptide chains; 50% of the substitutions are found in the N-terminal 32 residues [2]. Because the changes show these characteristics, and because of the generally large divergence, the structures and arrangements of further distantly related hemoglobins are of interest.

Studies on hemoglobin from the tropical lizard *Uromastix hardwickii* are now reported. This hemoglobin is of interest also because isolation of its constituent chains was found to be unexpectedly difficult. Chain separations were studied, and tryptic peptides corresponding to large parts of the amino acid sequence were determined. Results show that *Uromastix* hemoglobin has a roughly 50% positional identity with that of viper, indicating large evolutionary changes between these two reptilian species. In addition, evidence for the presence of multiple types of α- and β-chains was detected, explaining the difficulties encountered with chain separations.

2. MATERIALS AND METHODS

2.1. *Isolation of hemoglobin and carboxymethylation*

Blood was collected in 3.8% sodium citrate from *Uromastix hardwickii* caught wild in Pakistan. Red cells were separated by centrifugation, washed 3 times with physiological saline, lysed with distilled water, lyophilized, and used for globin preparation [5]. For carboxymethylation, the globin was reduced with dithiothreitol (185 nmol/mg protein; 2 h, 37°C), and reacted with neutralized labelled iodoacetic acid (1.26 μmol/mg protein, Radiochemical Centre, Amersham) for 2 h at 37°C

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in the dark. Reagents were removed by extensive dialysis against water.

2.2. *CM-Cellulose chromatography and counter current distribution*

Chain separations were tested by CM-cellulose chromatography [6] on Whatman CM 52 in 8 M urea with salt gradients, and buffers over pH 4.6–7.6. At pH 4.6 and 5.6, 8 M urea, 0.02 M Na acetate, 0–0.2 M NaCl was used; at pH 6.6 and 7.6, 8 M urea, 0.005–0.03 M sodium phosphate buffer. Fractions eluted were pooled, dialyzed against 5% acetic acid and desalted on a Sephadex G-50 column (1.5 × 180 cm) in 30% acetic acid.

For counter-current distribution [7], 60 transfers were performed in the solvent system secondary butanol:0.5 M acetic acid: 6% dichloroacetic acid (9:10:1, by vol.). Protein concentrations were followed by absorbance at 280 nm.

2.3. *Structural analysis*

TPCK-trypsin (Worthington) was used at enzyme:protein ratios of 1:50 in 0.1 M ammonium bicarbonate for 4 h at 37°C. Peptides were fractionated on Sephadex G-50 (1.5 × 180 cm) in 30% acetic acid, before purification by reversed-phase, high-performance liquid chromatography in a 0.1% trifluoroacetic acid/acetonitrile system [8]. Amino acid compositions were determined with a Beckman 121 M analyzer, after hydrolysis in evacuated tubes for 24 h at 110°C with 6 M HCl containing 0.5% phenol. Amino acid sequences were analyzed by manual degradations with the DABITC method [9,10], and by liquid phase sequencer degradations in the presence of pre-cycled polybrene [11] using high-performance liquid chromatography for identification [12].

3. RESULTS

3.1. *Fractionation of* Uromastix *globin preparations*

After removal of the heme group, the globin was chromatographed on carboxymethyl cellulose in 8 M urea, 0.02% mercaptoethanol, at different pH values over pH 4.6–7.6, as in section 2.2. The result of the separation at pH 5.6 is shown in fig. 1A. No resolved peaks are observed, and N-terminal sequence analysis revealed the presence of both α- and β-chains in all fractions. Resolution patterns and results were similar at all pH values and buffer conditions tried. Counter current distribution of the carboxymethylated globin preparation in secondary butanol:0.5 M acetic acid:6% dichloroacetic acid also gave incompletely separated fractions (fig. 1B), similarly found to be composed of mixtures of both α- and β-chains. It is concluded that clear α/β chain separations cannot be obtained in *Uromastix* globin preparations with methods effective for other globin preparations [6,7]. Apparently, multiple forms are present of both chains, explaining the patterns.

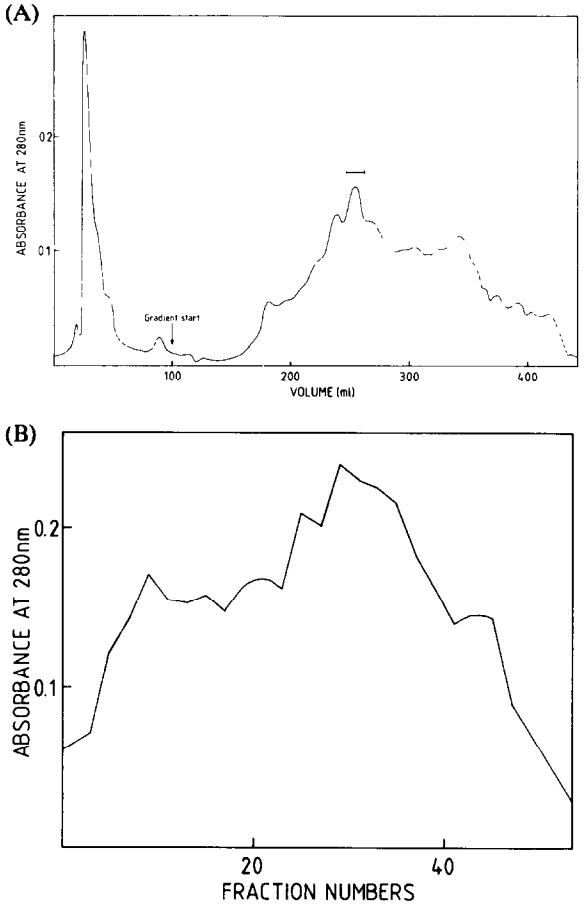

Fig.1. Fractionations of globin from *Uromastix hardwickii* (A) Chromatography on CM-cellulose (1.5 × 15 cm, flow 20 ml/h) in 8 M urea, 0.02% 2-mercaptoethanol, 0.02 M sodium acetate (pH 5.6) with a gradient of 0–0.2 M NaCl starting at the arrow. (B) Counter-current distribution of the carboxymethylated protein after 60 transfers in the solvent system 2-butanol: 0.5 M acetic acid: 6% dichloroacetic acid (9:10:1, by vol.).

Table 1

Results of liquid-phase sequencer analysis of the major fraction from the *Uromastix* globin on carboxymethyl cellulose chromatography

α:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Val	Leu	Thr	Asp	Asp	Asp	Lys	(Asn)	His	Val	Xxx	Ala	Ile	Trp	Gly
	(Σ:25)	15								11		11	10		
β:	Val	His	Trp	(Thr)	Ala	Glu	(Glu)	Lys	Ala	Leu	Ile	Asn	Ala	Tyr	Trp
	(Σ:25)								8	7	7			4	

The fraction is indicated by a bar in fig.1A. Values show nanomoles of stable residues recovered from application of a total of 30 nmol globin chains. Repetitive yield, 94%. The two alternatives in each cycle were obtained in mixture but are given and could be ascribed as α and β, respectively, after subsequent purification of peptides A1, A8, B19 and B22, from the N-termini of the corresponding chains (cf. fig.3). Approximate ratio of α:β-chains is 60:40, as judged by the ratio of residues in cycle 10, and of recoveries between cycles 1 and 2. Residues within parentheses were recovered in low yield. Similar degradation of unfractionated globin gave identical results, except for additional residues from cycle 3, and a higher α:β-chain ratio (approaching equality)

However, other proteins seem not to contribute, since all major fractions revealed essentially only structures typical for α- and β-chains (below).

3.2. *N-terminal structures*

Liquid-phase sequencer analysis of the fraction indicated by the bar in fig. 1A gave two major residues in each cycle for the first 15 positions as shown in table 1. The structures are as expected for α- and β-chains by homology. Degradations of the intact globin preparation revealed similar results for the first 14 cycles but increasing amounts of additional residues from position 3. This result is compatible with preparations composed essentially only of globin but containing chain multiplicity. Consequently, to gain further information on the possible homology with other globins, the preparation was analyzed by studies of the peptides generated by cleavages with trypsin.

3.3. *Internal structures*

The tryptic digest of the carboxymethylated globin was pre-separated into two fractions (A and B, mainly composed of large and small peptides, respectively) by chromatography on Sephadex G-50, and peptides from both fractions were purified by reversed-phase high-performance liquid chromatography. The pattern from fraction A is shown in fig. 2. Peptides purified were submitted to sequence analysis by manual degradations. The results are summarized in fig. 3, and supporting total compositions are given in table 2. Most structures obtained could be directly positioned in regions of α- and β-chains by homology with known structures of these chains from other species. The validity of some assignments of this type may be uncertain, if homology is low or peptides are short (cf. legend fig. 3), but the likely interpretations of all results are given in fig. 3.

No peptides corresponding to the C-terminal region of the α-chain (after position 90) were recovered (cf. fig. 3). This is probably merely

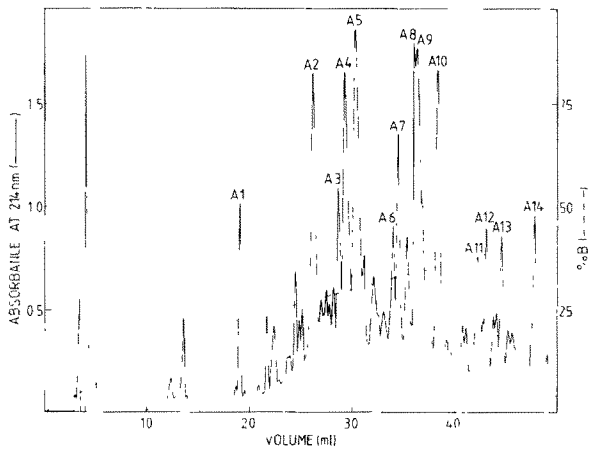

Fig.2. Purification of tryptic peptides by reversed-phase high-performance liquid chromatography on μ-Bondapak C_{18} in 0.1% trifluoroacetic acid with a gradient (%B, dashed line) of acetonitrile; flow, 60 ml/h). The separation shown is that from the first pool of the two (A and B) obtained by pre-fractionation of the complete globin digest on Sephadex G-50. Peptides reported in fig. 3 are numbered.

Table 2

Amino acid compositions of tryptic peptides purified

Peptide	A1	A2	A3	A8	A9	A10	A12	B15	B16	B18	B19	B20
Composition												
Asx	3.7 (4)	1.5 (2)	–	2.1 (2)	3.0 (3)	4.0 (4)	4.9 (5)	–	3.1 (3)	–	–	1.0 (1)
Thr	1.0 (1)	1.3 (1)	–	–	1.2 (1)	1.1 (1)	1.1 (1)	–	–	1.0 (1)	1.0 (1)	1.1 (1)
Ser	–	1.7 (2)	–	1.1 (1)	2.1 (2)	2.2 (2)	2.1 (2)	–	–	1.0 (1)	–	–
Glx	–	0.7 (1)	–	2.2 (2)	–	1.0 (1)	1.3 (1)	–	–	–	1.8 (2)	1.8 (2)
Pro	–	0.9 (1)	–	1.0 (1)	1.1 (1)	2.0 (2)	–	–	–	0.7 (1)	–	1.0 (1)
Gly	–	0.8 (1)	1.0 (1)	2.2 (2)	3.1 (3)	1.2 (1)	1.5 (1)	–	–	–	–	–
Ala	–	2.0 (2)	3.0 (3)	4.1 (4)	3.0 (3)	1.2 (1)	3.1 (3)	–	–	2.0 (2)	1.2 (1)	–
Val	1.8 (2)	–	2.5 (3)	1.2 (1)	–	–	2.8 (3)	–	1.0 (1)	–	1.1 (1)	1.0 (1)
Ile	–	–	–	0.9 (1)	1.9 (2)	1.0 (1)	0.8 (1)	–	–	–	–	–
Leu	0.9 (1)	0.9 (1)	2.0 (2)	1.0 (1)	–	2.0 (2)	3.0 (3)	–	1.0 (1)	1.0 (1)	–	–
Tyr	–	0.7 (1)	–	1.1 (1)	–	–	–	1.0 (1)	–	–	–	0.8 (1)
Phe	–	1.5 (2)	–	0.9 (1)	2.9 (3)	2.9 (3)	–	–	–	0.9 (1)	–	0.9 (1)
Lys	1.1 (1)	1.1 (1)	–	–	1.3 (1)	0.9 (1)	1.7 (2)	–	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)
His	0.9 (1)	1.1 (1)	1.0 (1)	1.0 (1)	–	–	–	1.0 (1)	–	1.0 (1)	0.9 (1)	–
Arg	0.8 (1)	–	0.9 (1)	1.0 (1)	–	–	–	1.0 (1)	–	–	–	–

Values shown are molar ratios from single hydrolyses without corrections for slow release, decomposition or contamination

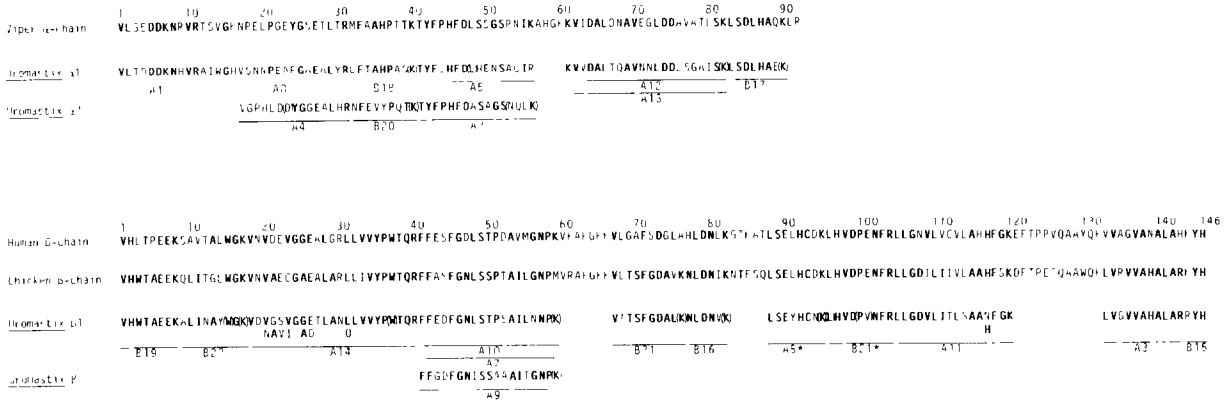

Fig.3. Summary of sequence results obtained from tryptic peptides of *Uromastix hardwickii*, hemoglobin. For ease of identification, all results are given below positions that are homologous in known hemoglobin chains of other species. Residues are given in the one-letter code, and were determined by the DABITC method, parentheses show low recoveries or conclusions from total compositions only. Supporting compositions are given in table 2 for peptides recovered pure. All peptide orders are listed by homology only, without isolation of overlapping fragments, and empty spaces in the *Uromastix* line indicate positions not analyzed. When more than one peptide matches the same region, all alternatives isolated are given below each other. For peptides A11 and A14, two alternatives were not isolated as separate peptides but detected as residue alternatives at positions indicated by double residue assignments. Positional identities with *Uromastix* residues are shown by heavy style. Positions beyond 92 in the α-chain are not included because no corresponding peptides were found. Peptides are given by a letter (A, B) to indicate origin from either of two pools upon Sephadex pre-fractionation and a number (1–14 for A; 15–22 for B) according to elution order from reversed-phase high-performance liquid chromatography (fig.2). Two peptides shown by asterisks were contaminants of major fragments with corresponding names.

because not all peptides were recovered. However, in relation to possibly lower recovery of C-terminal segments, it may be of interest that these parts of some hemoglobin chains appear susceptible to proteolytic removal [13]. Regions of both chains were now recovered in more than one peptide from the *Uromastix* preparation. For example, peptides A2 and A5 (fig. 3) are homologous, both corresponding to positions 41–56 of the viper α-chain, and peptides A9 and A10 both appear to correspond to a region around position 50 of the β-chain. Relative amounts differ but, from the peak heights in fig. 2, none of these peptides can be eliminated as an obvious contaminant. Consequently, the preparation is concluded to contain at least two types of both α- and β-chain, explaining the difficulties in attempts at chain separations.

4. DISCUSSION

Peptides determined with the present methods represent a selection of those of moderate size which were recovered in good yield. Nevertheless, they are sufficient to allow conclusions about heterogeneity, and about homologies of the *Uromastix* hemoglobin preparation.

4.1. *Heterogeneity*

No clear chain separations were obtained (fig. 1), although preparations are of acceptable purity in relation to hemoglobin, as judged from direct sequence analysis (table 1, legend). Some of the heterogeneity may be due to desamidations and other secondary chemical modifications. However, the peptide analyses also reveal that more than one peptide matches single regions in the hemoglobin chains (fig. 3). Some of this multiplicity is explained by alternative enzymatic cleavages (each of the peptide pairs A7/A9 and A12/A13, cf. fig. 3). However, remaining pairs have unique peptides that are homologous to hemoglobin chains. The globin homology of both alternatives, as well as the peak heights in fig. 2, suggest that both alternatives represent true hemoglobin chains. Relative amounts are difficult to estimate from the separation patterns, but top alternatives in fig. 3 appear to be major constituents (cf. fig. 2 where peak areas are: A7 + A10 > A9 for the peptides corresponding to position 50 in the β-chain, fig. 3). This conclusion is compatible with the results of direct sequence analysis of the whole preparation, showing two chains, α and β, most clearly (table 1) but also additional alternatives. Second alternatives were not recovered from all regions, although one was purified from most segments (fig. 3) and most major peptides were analyzed (fig. 2). Therefore, although additional heterogeneity may exist, the peptides purified appear representative. Hence, the results indicate the presence of more than one chain of each type in *Uromastix* hemoglobin preparations; at least two alternatives are apparent and one is of probably minor occurrence.

The differences are surprisingly large between the two alternatives within each chain type (around 50%, cf. fig. 3). Therefore, if the multiplicity of each type is due to corresponding *Uromastix hardwickii* gene multiplicity, the ancestral gene duplications must be distant. Alternatively, the species is not well defined and the variations detected might include some species or strain multiplicities (globin studied was a pool from 200 wild, adult animals). In any event, the results reveal structural heterogeneity in *Uromastix* globin preparations apart from establishing the extent of a general relationship between *Uromastix* and other hemoglobins.

4.2. *Homology*

The present results show that the hemoglobin chains studied are homologous with those of the other species in fig. 3 (roughly 50% positional identities). The relationships hardly appear closer to viper than to birds or mammals. This establishes that evolutionary changes even among reptilian species are large, and confirms conclusions [1,2,4] that hemoglobin is a protein with comparatively rapid rate of mutational alterations.

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