STRUCTURAL STUDY OF THE α CHAIN OF ONE HAEMOGLOBIN FROM THE ADULT SALAMANDER, *PLEURODELES WALTLII*

M. FLAVIN, Y. BLOUQUIT* and J. ROSA*

*Laboratoire de Biologie Générale. Université Paul Sabatier. 31000 Toulouse, and *Unité de Recherches sur les Anémies. INSERM U. 91, Hôpital Henri Mondor 94010 Creteil, France*

Received 5 March 1976
Revised version received 3 May 1976

1. Introduction

Relatively little information is available on the haemoglobins of the Urodeles. However Coates et al. [1] have recently reported the sequence of the α chain of *Taricha granulosa.*

The present paper describes the amino acid composition of an α chain of one Hb from *Pleurodeles waltlii* and the composition of its tryptic peptides. We have attempted to align the peptides using the sequence of the α chain of *Taricha granulosa* as a model.

The comparison of the primary structure of this α chain with homologous chains of other vertebrates will provide phylogenic informations. Furthermore, this α chain is present only in adult Hbs. It can be used as a marker to study the mechanisms of the ontogenic switch of *Pleurodeles* Hbs occurring during metamorphosis [2].

2. Materials and methods

Blood was obtained from adult *Pleurodeles waltlii* by cardiac puncture. We used pooled samples from different animals of the same brood, after checking the absence of haemoglobin heterogeneity from one individual to another (both in siblings and in unrelated animals of the same species). Lysis was effected with the use of digitonin [3] which facilited a higher recovery of haemoglobin.

Analytical electrophoresis of the hemolysate was carried out on cellulose acetate strips, using a Tris-EDTA-boric acid discontinuous buffer at pH 8.6 [4]. Haemoglobin was purified either by starch block electrophoresis [4] or by DEAE-Sephadex chromatography [5] using a stepwise NaCl gradient from 0.03 M and 0.12 M.

Globin was prepared by precipitation with cold acidified acetone [6]. Electrophoretic analysis of the chains was performed on cellulose acetate strips employing a 6 M urea buffer. The chains were isolated by chromatography in urea according to Clegg et al. [7], and alkylated under nitrogen in 8 M urea solution using iodoacetic acid as described by Crestfield et al. [8].

The amino acid composition of the α chain was established on a Jeol JLC 5 AH amino acid analyser. The manual Edman degradation described by Hermodson [9] was used to determine the N-terminal residues.

The carboxymethylated chain was digested with trypsin (TCPK-treated, Worthington) and maps of the tryptic peptides were made on silica gel thin-layer plates (Schleicher and Schüll). Peptides were isolated by preparative finger printing [10] and were eluted and hydrolysed for 24 h in constant boiling 6 N HCl in sealed tubes. They were dried and their amino acid composition determined.

3. Results

The electrophoretic pattern obtained from the haemolysates at pH 8.6 is shown in fig.1. Three bands are visible from the anode to the cathode. Bands A and B are present in approx. equal amounts,

North-Holland Publishing Company – Amsterdam

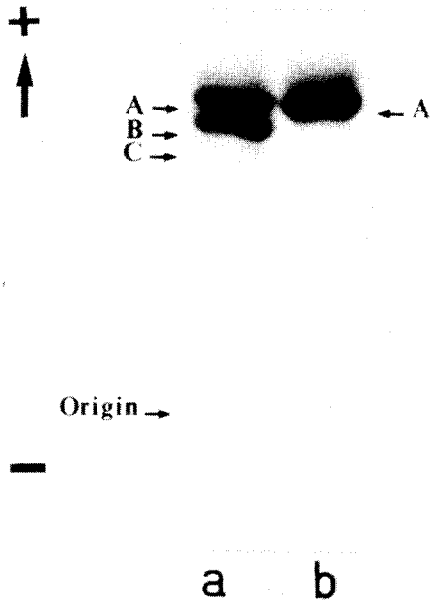


Fig.1. Electrophoresis of haemoglobins on cellulose acetate strips stained with amido black 10 B after application of 7 V/cm for 1.5 h. Electrophoresis was performed in a Tris-EDTA-borate discontinuous buffer containing 0.025 M boric acid, 0.05 M Tris and 0.7 mM EDTA (pH 8.6) for the bands and 0.0031 M boric acid for the tanks. (a) Whole hemolysate. (b) Fraction A purified.

while band C is a minor component. The same pattern was obtained upon starch block electrophoresis at pH 8.6. Fraction A was obtained in a pure form by this method or by DEAE-Sephadex column chromatography. Column chromatography of globin A on CM-cellulose in 8 M urea buffer demonstrated a chromatographic pattern (fig.2) very similar to that obtained from human A globin. The globin contained in the α peak showed only one band by electrophoresis in 6 M urea buffer.

By the Edman method, the α chain was found to have a methionine N-terminal group while the N-terminal group of the β chain was blocked. Use of carboxypeptidase B revealed the C-terminal sequences of the α chain as Lys–Tyr–Arg-COOH and Lys–Tyr–His-COOH for the β chain. The amino acid composition of the carboxymethylated α chain is given in table 1 and the finger print of the soluble tryptic peptides is shown in fig.3. Table 2 gives the amino acid composition of the tryptic peptides reported in the hypothetical sequence.

Table 1
Amino acid composition of the α chain of Pleurodeles waltlii

Amino acid	Observed	Nearest integer
Lysine	12.9	13
Histidine	10.0	10
Arginine	4.7	5
CM Cysteine	1.6	2
Aspartic acid	13.4	13
Threonine[a]	6.9	7
Serine[a]	9.6	10
Glutamic acid	7.5	8
Proline	4.4	4 or 5
Glycine	5.3	5
Alaline	15	15
Valine[b]	10.8	11
Methionine	3.7	4
Isoleucine	5.8	6
Leucine	13	13
Tyrosine	6.1	6
Phenylalanine	6.1	6
Tryptophane[c]		1

[a] Extrapoled to zero time from results at 24 and 40 h.
[b] Value at 72 h only.
[c] Determined by specific staining on finger prints.

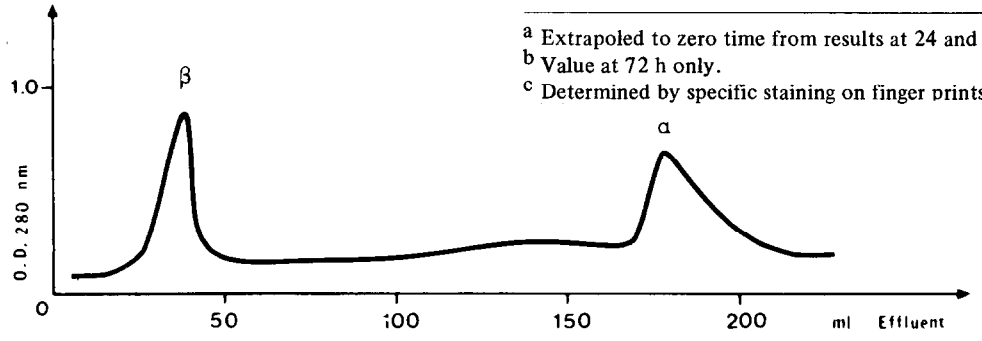


Fig.2. Chromatography of purified fraction A globin on CM cellulose in 8M urea containing 0.05 M 2-mercaptoethanol. The chains were eluted using a linear Na^+ ion gradient (0.005 – 0.03 M Na_2HPO_4).

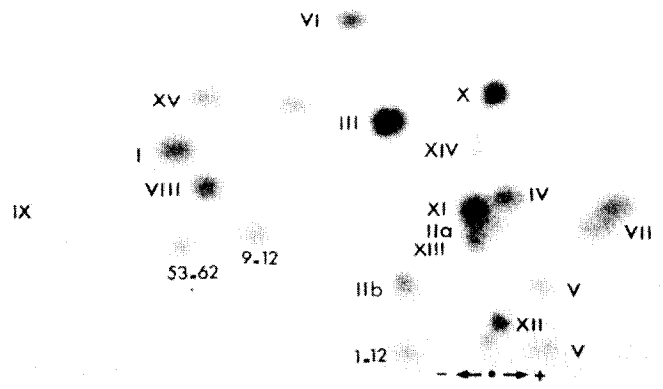


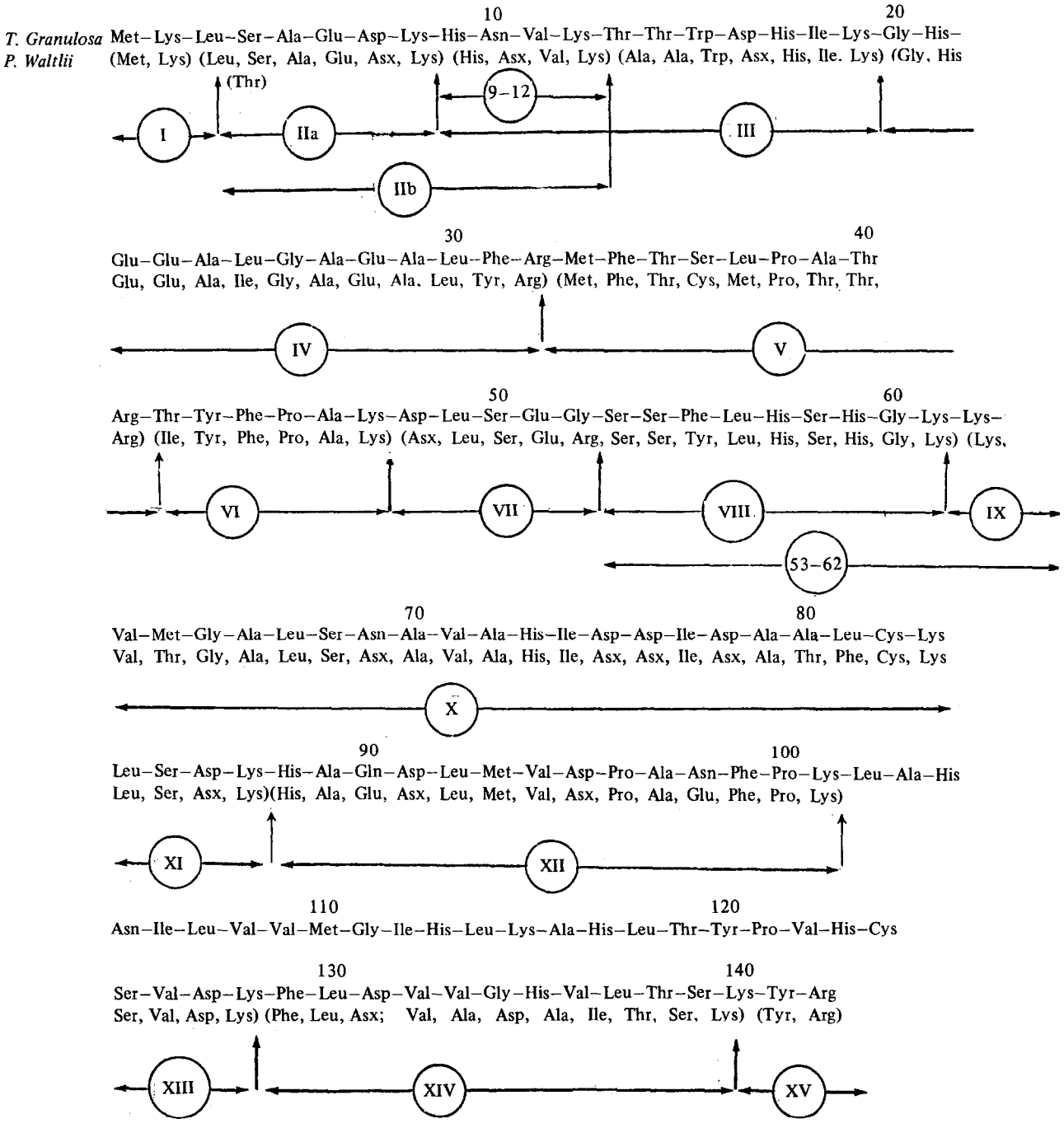
Fig.3. Fingerprint of the tryptic digest of the carboxymethylated α chain on a silica gel thin-layer plate. The tryptic peptides were resolved by electrophoresis at pH 6.4 in pyridine–acetic acid–water (100/4/1000 by vol) for 135 min and then by ascending chromatography in *n*-butanol–acetic acid–water–pyridine (400/80/210/320 by vol). Peptides were located with 0.02% ninhydrin. The numbering of peptides in roman figures correspond to their order in the proposed sequence. The arabic figures indicate overlapping sequence.

Table 2
Amino acid composition of tryptic peptides from *Pleurodeles* α chain

Peptide No.	I	IIa	IIb	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
Lys	1.12	0.87	1.72	1.28			1.16		1.2	1	1.02	1.02	1.18	0.96	1.07	
His			1.05	1.84	1.07				2.1		0.74		0.60			
Arg					1.01	0.90		0.87								1.08
CM Cys[a]						1							1			
Asp		1.21	2.27	2.02				0.92			3.6	1.08	2.25	1.13	2.22	
Thr			1.07			2.06					1.94				1.19	
Ser		0.71						0.96	2.8		1.07	1		0.77	1.16	
Glu		1.07	1.21		3.41			1.1					2.27			
Pro						0.9	1.15						2.16			
Gly					1.44				1.1		0.94					
Ala		1.28	1.12	1.88	3.58		1.14				3.72		2.15		2.29	
Val			0.70	0.97							1.45		0.96	1.13	1.02	
Met	0.88												1			
Ile				1	0.89		0.72				1.5				0.91	
Leu		0.86	0.85		1.2			1.07	0.95		1.02	0.90	1.15		0.84	
Tyr					0.92		0.69		0.85							0.92
Phe						1.03	1.14				1.02		1.27		0.85	
Trp[b]				1												
Recovery %	100	20	20	44	68	26	100	60	100		60	80	28	20	28	80

[a] Tryptophan detected in eluate from peptides maps. This amino acid was determined by specific staining of the analytical finger print as described by Easley [13]. The recoveries are expressed relative to those of peptides I and VI which were obtained in the highest yield. The absolute yield of peptide I based on the amount of starting globin was 18%.
[b] Carboxymethyl cystein.

Table 3
Alignment by homology of *Pleurodeles waltlii* α chain with *Taricha granulosa* α chain



The sequence of *Taricha granulosa* α chain was determined by Coates M., Brimhall B., Stenzel P., Hermodson M., Gibson D., Jones R. T., Vedvick T. (Private communication).

4. Discussion

The haemoglobin chain of *Pleurodeles waltlii* which is described in this paper is of the α type as demonstrated by its chromatographic behaviour on CMC column chromatography in urea buffer, by its C terminal sequence Arg–Tyr and by its amino acid composition.

We have compared the α chain of *Pleurodeles* with that of *Taricha granulosa* since these two species are closely related; the sequence of the α chain of *Taricha* has recently been reported by Coates et al. [1]. Many of the peptides of the *Pleurodeles waltlii* α chain are similar in amino acid composition to the peptides from *Taricha granulosa* haemoglobin. The residues were aligned by homology with the α *Taricha* chain and their hypothetic sequence is shown in table 3. Thus, the greatest part of the sequence could be reconstituted and the only part of the chain for which no peptide suitable for alignment was found was that from residues 102 to 124 in the *Taricha granulosa* sequence. According to Coates [1] this zone contains many insoluble residues; the corresponding peptides in *Pleurodeles* chain probably remained in the core.

Among 118 of the amino acids of the *Taricha* α chain which can be aligned with *Pleurodeles* peptides, only 16 are substituted and 2 are missing in the homologous sequence in *Pleurodeles.* These amino acids are known to be variable residues [11] at locations of frequent mutation.

The segments of the *Taricha* α chain composed by residues 1 to 12 and 53 to 61 are overlapped by two or more peptides of *Pleurodeles.* This is easily explained in most cases by incomplete tryptic cleavages. The duplication of peptide V (fig.3) can be explained by the oxidation state of the methionine residue at position 33 or 37 [12].

Peptide IIa which contains residue 3 to 8 has one serine residue which corresponds to position 4 according to the α *Taricha* chain. In the peptide IIb which corresponds to the sequence 3 to 12, the serine residue at position 4 is replaced by a threonine residue. This heterogeneity could be explained by the hypothesis that the α chain is heterogenous; i.e. one chain possesses a serine residue and another a threonine residue in position 4. In the peptide IIb there is no cleavage after Lys 8 probably because of the vicinity of an Asp residue with a negative charge. In the other chain containing Ser in position 4, residue 7 is perhaps an asparagine and thus cleavage occurs after Lys 8. In this hypothesis, an opposite charge must exist in another part of the chain in order to explain the same behaviour of both chains on electrophoresis and chromatography.

According to these findings, two α chains which differ in a small number of residues and which cannot be separated by the usual techniques were probably present in fraction A. The sequence of these peptides is however needed to support this hypothesis.

Upon comparison of the α chain of *Taricha granulosa* and *Pleurodeles waltlii* some important similarities are evident. Thus, there are 18 differences between the 118 amino acids in the sequences of *Taricha* and *Pleurodeles.* The supposed rate of 1% mutation every 5 million years for amino acids of mammalian haemoglobins [11] implies an evolutionary divergence between *Pleurodeles* and *Taricha* some 75 million years ago (15% difference) i.e. near the end of mesozoic era. Such a divergence is consistent with geological data.

Acknowledgements

We thank Dr R. T. Jones for kindly communicating his results on the sequence of α chain of *Taricha granulosa* and Dr Chapman for careful assistance in reviewing the manuscript.

This work was supported by le Centre National de la Recherche Scientifique (ATP No. 1884) and la Délégation Générale à la Recherche Scientifique et Technique (grant No. 73 7 1259) and by l'Institut National de la Santé et de la Recherche Médicale.

References

[1] Coates, M., Brimhall, B., Stenzel, P., Hermodson, M., Gibson, D., Jones, R. T. and Vedvick, T., private communication.
[2] Flavin, M., Gasser, F. and Beetschen, J. C. (1973) C. R. Acad. Sci. 275, Série Dn 2411.
[3] Coates, M., private communication.
[4] Hall, J. G. and Motulski, A. G. (1968) Nature 217, 569.
[5] Huisman, T. H. J. and Dozy, A. M. (1965) J. Chromatogr. 19, 160.

[6] Rossi-Fanelli, A. R., Antonini, E. and Caputo, A. (1958) Biochim. Biophys. Acta 30, 608.
[7] Clegg, J. B., Naughton, M. A. and Weatherall, D. J. (1966) J. Mol. Biol. 19, 91.
[8] Crestfield, A. M., Moore, S. and Stein, W. H. (1963) J. Biol. Chem. 238, 622.
[9] Hermodson, M. A., Kuhn, R. W., Walsh, K. A., Neurath, H., Eriksen, N. and Benditt, E. P. (1972) Biochemistry 11, 2934.
[10] Braconnier, F., Beuzard, Y., El Gammal, H., Garel, M. C. and Rosa, J. (1975) Nouv. Rev. Fr. Hémat. 15, 527.
[11] Dayhoff, M. O. (1969) Atlas of protein sequence and structure Vol. 4, Silver Spring. The National Biomedical Research Foundation.
[12] Baglioni, C. and Ingram, V. M. (1961) Biochim. Biophys. Acta 48, 235.
[13] Easley, C. W. (1965) Biochim. Biophys. Acta 107, 386.