ISOLATION OF COELACANTH (*LATIMERIA CHALUMNAE*) MYOGLOBIN

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1. Introduction

The coelacanth *Latimeria chalumnae* is the only living species of Crossopterygii, ancestors of land vertebrates which have diverged from the other bony fishes some 500 million years ago [1]. On the other hand the modern representative of coelacanths looks very much like the restorations of its Mesozoic forebears [2].

It is of great interest at the point of view of evolution to compare coelacanth proteins with those of lungfishes and amphibians. Lungfishes and crossopterygians are supposed to arise from a common bony-fish stock and to have diverged from common ancestors [2]. It has been found, on the other hand, that African lungfishes have amphibian neurohypophysial hormones and not current bony fish hormones [3]. Furthermore the comparison of coelacanth proteins with those of other vertebrates might also reveal differences in the rates of evolution of proteins.

Hemoglobin and myoglobin are good evolutionary tracers. The amino acid sequences of several mammalian hemoglobins and myoglobins are fully known [4], and among amphibians, the β-chain of frog (*Rana esculenta*) hemoglobin has recently been characterized [5]. Myoglobin is present in fin muscles of coelacanth and therefore a comparative structural study can be carried out.

2. Methods and materials

2.1. *Purification of coelacanth myoglobin*

A male coelacanth, fished in the vicinity of Moroni (Great Comoro Island) was frozen about 6 hr after capture and flown frozen to the laboratory. The specimen was allowed to thaw slowly and uniformly during about 48 hr at 5°. At the time of dissection, some ice crystals remained in the depths of the body cavity (the liquid of the notochordal canal was still frozen) and the condition of the tissues seemed very good.

Coelacanth fins are mounted on pedicles (except first dorsal and caudal) and have red muscles [1]. Myoglobin was prepared from 138 g of red muscle obtained from the left pelvic fin. The tissue was homogenized with 150 ml of 0.05 M Tris buffer, pH 7.2, for 30 sec at 5° and the red solution was separated by centrifuging. A second extraction was carried out in the same way and the supernatant was added to the first solution (final volume: 280 ml).

Myoglobin was first purified by fractionated precipitation with ammonium sulfate: the precipitate at 0.7 sat. (6 hr, 5°) was removed by centrifugation and the material precipitated between 0.7 and 0.9 sat. (18 hr, 5°) was collected, dissolved in 10 ml of water and dialysed against water for 24 hr. Myoglobin was then isolated by chromatography on carboxymethyl-Sephadex C-50. A column (2 × 15 cm; particle size 40–120 μ) equilibrated with 0.05 M sodium phosphate buffer pH 6.0 was used. Contaminating proteins passed through the column but myoglobin was adsorbed at the top; it was eluted with a 0.3 M phosphate buffer pH 6.0. The material, dialysed against water for 24 hr, was freeze-dried (weight: 49 mg).

2.2. *Control of homogeneity*

2.2.1. Analytical chromatography on CM-Sephadex with a linear gradient

3 mg of myoglobin were chromatographed on a column of CM-Sephadex C-50 (1 × 10 cm) equilibrated

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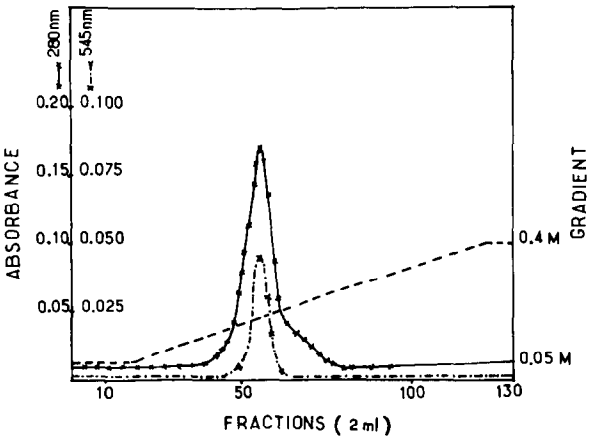

Fig. 1. Chromatography of coelacanth myoglobin on carboxymethyl-Sephadex C-50 (details in the text).

with a 0.05 M phosphate buffer pH 6.0 containing 0.1% KCN. After washing with 40 ml of buffer, a linear gradient of concentration (from 0.05 M to 0.4 M; 200 ml) was applied and 2 ml fractions were collected; protein and heme concentrations were determined by spectrophotometry at 280 nm and 545 nm, respectively. A single symmetric peak was observed (fig. 1).

2.2.2. Electrophoresis on polyacrylamide gel

Coelacanth myoglobin was subjected to a disc-electrophoresis on 7% polyacrylamide gel at pH 9.5 under the conditions described by Ornstein [6] and Davies [7]. Horse (2 X crystallized Fluka ref. 603 512) and sperm whale (1 X crystallized Fluka ref. 70 020) myoglobins were used for comparison. A single band was observed for coelacanth and sperm whale myoglobins but two for horse protein. Sperm whale myoglobin is more cationic than coelacanth myoglobin. Apomyoglobins were prepared by acetone precipitation at pH 3.0 and examined by electrophoresis on 7.5% polyacrylamide gel at pH 2.3. Some heterogeneity could be observed for commercial horse and sperm whale apomyoglobins but coelacanth apomyoglobin gave a single component.

2.2.3. Amino acid composition

Samples (50 μg) of coelacanth apomyoglobin were hydrolyzed in evacuated, sealed tubes with 6 N HCl for 24, 48 and 72 hr at 105°, and analyzed with a Spinco 120 B automatic analyzer fitted with a high-sensitivity cell [8]. The results are given in table 1. When compared to the known vertebrate myoglobins [9], coelacanth myoglobin is clearly richer in leucine and poorer in glycine; on the other hand, although yellowfin tuna myoglobin [10] has more alanine and isoleucine and fewer leucine and valine than coelacanth myoglobin, the sums of these residues are virtually equal. The latter comparison might be of interest because coelacanth and tuna belong to two different sub-classes of bony fishes which are supposed to have diverged 450–500 million years ago [2].

Table 1
Amino acid composition of coelacanth apomyoglobin* (values expressed as residues per mole with an assumed molecular weight of 16,000).

	24 hr	48 hr	72 hr	Average**	Assumed number of residues
Lys	15.25	16.02	15.80	15.69	16
His	10.14	9.56	9.71	9.80	10
Arg	3.73	3.73	4.43	3.96	4
Asp	10.95	10.94	10.87	10.92	11
Thr	9.60	10.14	8.94	9.60	10
Ser	8.30	7.85	7.41	8.30	8
Glu	14.18	14.53	13.90	14.20	14
Pro	3.89	3.11	3.33	3.44	3–4
Gly	5.88	4.57	5.51	5.32	5
Ala	12.21	11.62	12.04	11.95	12
Cys**	–	–	–	1.52	2
Val	11.12	11.93	11.92	11.92	12
Met	–	–	–	–	–
Ile	4.95	5.68	6.69	6.69	7
Leu	24.61	25.19	24.95	24.91	25
Tyr	3.50	3.44	3.76	3.56	4
Phe	7.41	7.50	7.77	7.56	8
Trp***	–	–	–	–	2
Total					153–154

* Amino acid analysis was performed in duplicate at each time.

** For Thr and Ser, the values are those of the 24 hr-hydrolysis and for Val and Ile those of the 72 hr-hydrolysis. Cys was determined as cysteic acid on a separate performic acid-oxidized sample with a 48 hr-hydrolysis.

*** Number of residues deduced from the number of tryptophan-containing peptides on the tryptic peptide map.

2.2.4. N-terminal amino acid sequence

The N-terminal sequence has been determined by using a SOCOSI Model PS 100 sequencer under the conditions described by Edman and Begg [11]. Merck reagents (kit 8012 for sequential analysis) were employed and the program was virtually identical to that described for humpback whale myoglobin [11]. Coelacanth apomyoglobin was used without any modification. Three assays were performed with 250, 500 and 500 nmole. Sperm whale and horse apomyoglobins were used as references. Phenylthiohydantoins of amino acids were identified by thin-layer chromatography [12]. The amino acid sequence of the first eight residues of coelacanth myoglobin Val–Leu–Trp–Ala––Ala–Asp–Glu–Thr was determined. Results were ambiguous after the eighth cycle.

3. Results and discussion

Coelacanth myoglobin showed a single component by analytical chromatography and disc-electrophoresis; furthermore a single residue was observed at each step of the Edman degradation so that the material can be regarded as homogeneous.

The N-terminal sequences of several mammalian myoglobins (man [13], gibbon [14], sperm whale [15], humpback whale [11], porpoise [16], dolphin [17], harbor seal [16], horse [18], beef [19], and kangaroo [20]) as well as that of chicken myoglobin [21] are known to date. It is of interest to note that if there are 4 invariant residues (no. 2, 3, 6 and 7) in the first eight positions of these myoglobins, after including coelacanth myoglobin in the family only the position no. 2 remains invariant. Eutherians and metatherians (Marsupiala) have diverged some 130 million years ago and nevertheless the N-terminal sequences of man and kangaroo myoglobins are identical for the first eight positions. There is one substitution in this part of the polypeptide chain between man and chicken myoglobins although the elapsed time since the divergence of mammalian and avian lines is about 300 million years [2]. By contrast, there are seven substitutions in the case of coelacanth myoglobin. It might be possible that the rate of myoglobin evolution was not uniform in all the vertebrates lines.

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References

[1] J. Millot et J. Anthony, Anatomie de Latimeria chalumnae, Tome 1, Centre National de la Recherche Scientifique (1958) Paris.
[2] A.S. Romer, Vertebrate Paleontology (The University of Chicago Press, Chicago, Ill., 1966).
[3] R. Acher, J. Chauvet and M.T. Chauvet, Nature 227 (1970) 186.
[4] M.D. Dayhoff, Atlas of Protein Sequence and Structure (The National Biomedical Research Foundation, Silver Spring, Md., 1969) Vol. 4.
[5] J.P. Chauvet and R. Acher, Biochemistry 11 (1972) 916.
[6] L. Ornstein, Ann. N.Y. Acad. Sci. 121 (1970) 321.
[7] B.J. Davis, Ann. N.Y. Acad. Sci. 121 (1970) 404.
[8] D.H. Spackman, W.H. Stein and S. Moore, Anal. Chem. 30 (1958) 1190.
[9] M. Dautrevaux, Recent Developments in the Chemical Study of Protein Structures, Colloque INSERM (1971) Paris.
[10] C.H.W. Hirs and H.S. Olscott, Biochim. Biophys. Acta 82 (1964) 178.
[11] P. Edman and J. Begg, European J. Biochem. 1 (1967) 80.
[12] J. Jeppsson and J. Sjöquist, Analytical Biochemistry 18 (1967) 264.
[13] A.E.R. Herrera and H. Lehmann, Nature New Biology 232 (1971) 149.
[14] A.E.R. Herrera and H. Lehmann, Biochim. Biophys. Acta 251 (1971) 482.
[15] A.B. Edmunson, Nature 205 (1965) 883.
[16] R.A. Bradshaw and F.R.N. Gurd, J. Biol. Chem. 244 (1969) 2167.
[17] M. Karadjova, P. Nedkov, A. Bakardjieva and N. Genov, Biochim. Biophys. Acta 221 (1970) 136.
[18] M. Dautrevaux, Y. Boulanger, K. Han and G. Biserte, European J. Biochem. 11 (1969) 267.
[19] K. Han, M. Dautrevaux, X. Chaila and G. Biserte, European J. Biochem. 16 (1970) 465.
[20] G.M. Air, O.P. Thompson, B.J. Richardson and G.B. Sharman, Nature 229 (1971) 391.
[21] M. Deconinck, J. Depreter, C. Paul, S. Pfeiffer, A.G. Schnek, F.W. Putnam and J. Leonis, FEBS Letters 23 (1972) 279.