Structural comparison of the haemoglobin components of the armoured catfish *Pterygoplichthys pardalis**

Evolutionary considerations

Francesco BOSSA,†‡ Maria Rosaria SAVI,† Donatella BARRA,‡§ and Maurizio BRUNORI‡||
†Istituto di Chimica Biologica, Università di Roma, Città Universitaria, 00185 Roma, Italy, ‡Centro di Biologia Molecolare del C.N.R., Roma, Italy, §Istituto di Chimica Biologica, Università di Camerino, Camerino (MC), Italy, and ||Istituto di Chimica, Facoltà di Medicina, Università di Roma, Roma, Italy

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Amino acid analyses and peptide mapping were performed for the four main haemoglobins from the armoured catfish *Pterygoplichthys pardalis*; component I, which is functionally distinct from the others, is structurally unique, whereas components II, III and IV, functionally indistinguishable, are closely related in structure. Compositional difference indices are calculated for the four components and for the two major haemoglobins from the trout *Salmo irideus*, and the results are discussed in terms of structural relationships and evolutionary history of fish haemoglobins.

Haemoglobin multiplicity is of common occurrence in fish haemolysates, and in several instances the various components have distinctly different functional properties, which led to the hypothesis that multiplicity represents a mechanism of adaptation to cope with a variable environment (Brunori, 1975; Riggs, 1979).

Pterygoplichthys pardalis is probably the most common among the armoured catfish which populate the Amazon waters, and is very interesting from the standpoint of respiratory physiology because, in addition to gills, it is provided with an alternative O₂-exchange system associated with its gut, which makes it an optional 'air breather'.

For these reasons, during the 'Alpha-Helix' expedition in the Amazonas, the four haemoglobin components of *Pterygoplichthys* were separated and characterized from the functional point of view (Brunori *et al.*, 1979). It was shown that these haemoglobins can be divided in two groups characterized by distinct and complementary functional properties: one group is represented by the most abundant haemoglobin, component I, the other comprises components II, III and IV.

As a first contribution to unravelling the molecular basis of multiple functions in the haemoglobins of *Pterygoplichthys pardalis*, the present paper reports the amino acid analyses and the

tryptic-peptide maps of these four components. The differences in amino acid compositions were quantitatively evaluated and compared with those determined for the system of multiple haemoglobins present in trout (*Salmo irideus*) blood.

Materials and methods

Blood obtained from several specimens of *Pterygoplichthys pardalis* was collected during the Alpha-Helix expedition in the Amazonas (November–December, 1976) and the haemolysate was kept as an (NH₄)₂SO₄ precipitate under pure CO at –20°C. Separation of the four haemoglobin components was achieved as described by Brunori *et al.* (1979).

For amino acid analysis, samples (0.04 mg) of each protein were hydrolysed in 6 M-HCl (0.2 ml), containing a trace of phenol (Sanger & Thompson, 1963), under vacuum at 110°C for 24 h. Analyses were performed by using an LKB 4400 automatic analyser equipped with a Spectra-Physics System I computing integrator.

The compositional difference index used (Cornish-Bowden, 1979) is defined by:

$$S\Delta n = \frac{1}{2} \sum_i (n_{iA} - n_{iB})^2$$

where n_{iA} and n_{iB} are the number of residues of amino acid i in proteins A and B, and the summation was carried out for the types of amino acid that are readily distinguishable in composition measurements (thus aspartic acid and asparagine were

Abbreviation used: Pt Hb I (etc.), *Pterygoplichthys* haemoglobin component I (etc.).

* Dedicated to Professor A. E. Braunstein on his 80th birthday.

counted together, as were glutamic acid and glutamine).

Peptide maps were obtained after digestion of portions (3 mg) of carboxymethylated globins with trypsin [Worthington, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK')-treated] at an enzyme/substrate ratio of 1:50, at 37°C in 0.1 M-NH₄HCO₃ for 6 h; digestion was stopped by acidification with few drops of acetic acid and the peptide mixture was freeze-dried. Portions of the digests were dissolved in water at concentrations of about 1 µmol of αβ-dimer/ml, and 10 µl of this solution were spotted on to silica-gel thin-layer sheets (Baker-flex, IB2). Sheets were developed first by chromatography in chloroform/NH₃ (34%)/methanol (2:1:2, by vol.) and then by electrophoresis (Varsol-cooled tank;

Savant TLE20) at pH 6.5 (pyridine/acetic acid/water, 20:1:179, by vol.) for 45 min at 900 V. After air-drying, plates were stained with 0.1% ninhydrin in ethanol/acetic acid/collidine (15:5:2, by vol.).

Results and discussion

The amino acid compositions of the four haemoglobin components are given in Table 1. The difference between Pt Hb I on one hand, and the other three components (Pt Hb II, III and IV) on the other, is immediately apparent, whereas the amino acid compositions of Pt Hb II, III and IV are only slightly different from each other (significant variations being detectable, however, in the content of glutamic acid, alanine and valine).

The maps of the soluble tryptic peptides from the four components reveal a clear structural difference between Pt Hb I and the group composed of Pt Hb II, III and IV, although only minor differences were detected between the proteins from the latter group.

Values of compositional difference indices Δn were calculated for each pair of haemoglobin components from *Pterygoplichthys* haemolysate and the results are given in Table 2, where the estimated percentage sequence differences for each pair of proteins are also given. The indices, claimed to be unbiased estimators of the number of sequence differences between two related proteins, clearly express, in a quantitative way, the difference between Pt Hb I on one hand and Pt Hb II, III and IV on the other (the estimated percentage sequence differences ranging between 46.2 and 51.3%); they also show the close structural relationships within the group Pt Hb II, III and IV, with a minimum of estimated percentage sequence differences between Pt Hb III and IV (1.0%) and a maximum between Pt Hb II and IV (8.1%).

Comparison with trout haemoglobins

The haemolysate from trout (*Salmo irideus*) contains three major haemoglobin components with distinct structural and functional properties (Brunori, 1975). All components bind O₂ co-operatively at physiological pH; the more basic component, trout

Table 1. Amino acid composition of the haemoglobin components of *Pterygoplichthys pardalis*

Values are expressed as the number of residues per αβ-dimer.

Amino acid	Pt Hb I	Pt Hb II	Pt Hb III	Pt Hb IV
Aspartic acid	31.0	27.0	26.7	26.5
Threonine	10.1	9.8	9.4	9.2
Serine	22.0	25.1	24.6	24.7
Glutamic acid	15.1	21.4	22.0	23.0
Proline	11.0	12.0	12.3	12.6
Glycine	19.8	18.3	17.8	17.8
Alanine	38.8	27.5	29.3	30.5
Cysteine*	1.5	1.6	1.5	1.5
Valine	29.0	28.1	24.6	23.0
Methionine	1.9	0.3	0.2	0.2
Isoleucine	12.2	12.5	13.6	13.2
Leucine	31.1	36.6	37.7	38.0
Tyrosine	8.5	6.4	6.3	6.3
Phenylalanine	17.6	18.4	17.8	17.8
Histidine	9.4	15.0	15.7	15.5
Lysine	24.8	23.8	23.5	23.6
Arginine	7.9	7.5	7.8	8.1
Tryptophan†	5.4	4.7	6.2	5.5

* Determined as cysteic acid after hydrolysis in the presence of dimethyl sulphoxide (Spencer & Wold, 1969).

† Determined after hydrolysis with 4 M-methanesulphonic acid (Simpson *et al.*, 1976).

Table 2. Compositional difference indices (Δn) for all pairs of haemoglobins from *Pterygoplichthys pardalis* and the two major components from trout

Corresponding estimated percentage sequence differences are given in parentheses.

	Pt Hb II	Pt Hb III	Pt Hb IV	Trout Hb I	Trout Hb IV
Pt Hb I	134.1 (46.2)	142.0 (49.0)	148.7 (51.3)	85.7 (29.5)	151.3 (52.2)
Pt Hb II		10.4 (3.6)	23.5 (8.1)	271.9 (93.8)	167.2 (57.7)
Pt Hb III			3.0 (1.0)	278.5 (96.0)	152.0 (52.4)
Pt Hb IV				286.1 (98.7)	162.5 (56.0)
Trout Hb I					159.6 (55.0)

HbI, is characterized by the invariance of the O₂-binding curve with changes of proton and organic phosphate concentration, whereas trout Hb IV shows a very large pH-dependence of the O₂-binding curve (the Root effect). Therefore homotropic and heterotropic effects display similarities and differences among the various components of trout and *Pterygoplichthys* (see also Barra *et al.*, 1981) haemoglobin. Structural studies on trout haemoglobins at the level of primary structure are well advanced (Bossa *et al.*, 1978).

For these reasons, the trout system represents an obvious reference for comparison with the *Pterygoplichthys* haemoglobin system, also considering that the two fish belong to two different superorders (i.e. Protacanthopherygii for trout and Ostariophysi for *Pterygoplichthys*), and thus are distantly related. Therefore amino-acid-composition data for trout Hb I and IV, (D. Barra, unpublished work), obtained under experimental conditions identical with those adopted for *Pterygoplichthys* haemoglobins, were included in the comparison. Values of compositional difference indices were calculated for each pair, as indicated in Table 2. These comparisons indicate that the estimated sequence differences for the pair Pt Hb I/Pt Hb IV (51.3%) and for the pair trout Hb I/trout Hb IV (55.0%) are of the same order of magnitude; for the latter pair, direct sequence data allow a straightforward comparison of 83.0% of the structures, indicating a sequence difference of 34.7% (Bossa *et al.*, 1978; F. Bossa & D. Barra, unpublished work). This difference seems in general agreement with the statement (Cornish-Bowden, 1979) that the effect of experimental error in amino acid composition should lead to an increase in the value of Δn , which will overestimate sequence differences.

As far as the comparison between the two organisms is concerned, the estimated sequence difference for the pair of more basic haemoglobins, Pt Hb I and trout Hb I (29.5%), appears significantly less than that for the pair of more acidic components, Pt Hb IV and trout Hb IV (56.0%). It is noteworthy that none of the *Pterygoplichthys* haemoglobins displayed precipitating activity against an antiserum to trout Hb IV, which, however, did not react with any of the haemoglobins tested during the Alpha-Helix expedition (Reichlin & Davis, 1979). These data also show that the estimated sequence differences for haemoglobins from fish belonging to different superorders are of the same order of magnitude; even more important is the fact that the difference between two functionally similar components, i.e. Pt Hb I and trout Hb I (Brunori *et al.*, 1979), is considerably smaller than that observed between functionally distinct haemoglobins belonging to the same animal and actually present within the same erythrocyte (Brun-

ori *et al.*, 1974) (compare, in Table 2, trout Hb I with trout Hb IV and Pt Hb I with Pt Hb IV). These observations strongly support the conclusion reached from the comparison of the N-terminal sequences (62 residues) of trout I, trout IV and *Catostomus clarkii* (a catostomid fish) α -chains (Bossa *et al.*, 1978). When taken together with the more-limited, but still important, information derived from immunological studies (Reichlin & Davis, 1979), this set of results demands that evolutionary trees from structural information obtained on isoproteins (isoenzymes or multiple haemoglobins) present in the same animal and characterized by specialized functions should be taken with great caution. Presumably the multiple haemoglobin components present in many fishes, and in particular those from the Amazonian basin, may be taken as representative of systems in which both divergent and convergent evolution took place to a relevant extent. In fact all the tetrameric haemoglobins so far studied clearly share a common frame of fundamental structural and functional properties (leading to co-operative O₂ binding), and the selection of additional structural features involved in more specialized functions (heterotropic effects) was driven by the 'pressure' exerted by similar environmental characteristics on different, phylogenetically very distant, animals.

In fact, additional detailed structural information on multiple fish haemoglobins is necessary, in conjunction with advances in the phylogeny and classification, to provide an answer to a fundamental question in evolution, i.e. whether distinct haemoglobins (such as Pt Hb I and IV or trout Hb I and IV) evolved from a common ancestor molecule *before or after* divergence of the respective fish from the common ancestor fish, or whether the ability to perform similar specialized functions was included in the haemoglobin systems of *already diverged animals* by selecting for the same molecular mechanism or by arriving at the same functional performance employing different structural solutions.

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