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HAEMOGLOBIN OF THE LAMPREY, *LAMPETRA FLUVIATILIS*

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SUMMARY

The haemoglobin of the brook lamprey (*Lampetra fluviatilis*) has been investigated. It has a molecular weight of 17,200 and an absorption spectrum in the visible and Soret regions very similar to human haemoglobin. The tryptophan fine-structure absorption band in the u.v. and amino-acid composition of lamprey and human haemoglobins are significantly different. Lamprey haemoglobin has one sulfhydryl group per mole of native and 1.9 group per mole of denatured protein. All 26 specimens examined showed one minor and two major components on electrophoresis. Lamprey haemoglobin is of special interest in providing a clue to the evolution of haemoglobin in the vertebrates.

INTRODUCTION

Cyclostomes are the most primitive living vertebrates. Together with the extinct ostracaderms, the most ancient fossil vertebrates, they form the super-class *Agnatha*, which has followed an independent evolution since earliest vertebrate origins. Cyclostomes are also the most primitive animals known to have haemoglobin enclosed in erythrocytes. Haemoglobin and myoglobin are two proteins which have been intensively studied both from the structural and the functional point of view. Investigations of haemoglobins in different species[1] show how the protein has been modified in the course of evolution to fulfil the physiological needs of a wide range of organisms. The haemoglobin of the lamprey is of special interest since it lies intermediate in structure and properties between myoglobins and haemoglobins of higher

vertebrates, and so provides clues to the evolution of the whole group of proteins.

Although isolated studies of lamprey haemoglobin have been reported, there has been no systematic survey study of its properties. This paper reports the results of a series of investigations of the haemoglobin of the lamprey undertaken to that end.

MATERIAL AND METHODS OF STUDY

Twenty six adult specimens of the brook lamprey, *Lampetra fluviatilis*, and two specimens of the sea lamprey, *Petromyzon marinus*, were obtained commercially and bled into acid-citrate-dextrose solution. The washed red cells were lysed by addition of distilled water or exposure for 1 min to ultrasonic vibration from a magnetostriction transducer (25 kc/sec; 200 W) in an ice bath. Cell stroma was removed by centrifugation at 12,000 × *g* for 30 min. Electrophoresis was carried out in a starch block[2], using barbiturate buffer, pH 8.6; *I* 0.15, on 3 MM filter paper with the same buffer, and in agar using citrate buffer, pH 6.5 (see ref.[3]). Sedimentation was studied in phosphate buffer, pH 6.8, ionic strength 0.2, in a Spinco Model E ultracentrifuge at speeds of 16,200 rev./min for molecular weight determinations by the ARCHIBALD method[4,5], and at 59,780 rev./min for sedimentation measurements. Protein concentrations were 0.84 % and 0.42 %, respectively. The light filter was removed and exposure increased because of the strong absorption by the solutions; this made it impossible to obtain photographic records of the highest quality, but should not materially affect the accuracy of the results. Absorption spectra were measured in a Unicam S.P. 600 spectrophotometer, the trytophan fine-structure band being located by the moving-plate logarithmic cam spectrographic technique of HOLIDAY[6]. The concentration of haemoglobin solutions for titrations was measured spectrophotometrically, the millimolar extinction coefficient at 540 mμ being taken as 14.9 per atom of iron. The sulphydryl content of lamprey carboxyhaemoglobin, both native and denatured, was determined by amperometric titration with phenyl mercuric hydroxide using the dropping mercury indicator electrode as described by ALLISON AND CECIL[7] for adult human haemoglobin. Complete amino-acid determinations were made on 22- and 24-h hydrolysates, using the column chromatographic technique of MOORE AND STEIN as described elsewhere[8,9].

RESULTS

Molecular weight

SVEDBERG AND ERIKSON-QUESNEL[10] deduced from sedimentation and diffusion studies that the haemoglobin of the brook lamprey, *Lampetra* (= *Petromyzon*) *fluviatilis*, had a molecular weight of 19,100, whereas that of the hagfish, *Myxine glutinosa*, was 23,100. LENHERT, LOWE AND CARLSON[11], using ACHIBALD's method, reported a molecular weight of 23,600 for haemoglobin from *P. marinus*. Both groups obtained lower values from haem and iron analyses (17,250–18,600, assuming one haem per mole), and therefore concluded that there was some aggregation into dimers. We have repeated the molecular weight determination by ARCHIBALD's method[4,5] on haemoglobin purified by electrophoresis in starch blocks. The mean value, from five exposures taken between 30 and 60 min after reaching speed (16,200 rev./min) was 17,200, which is consistent with the observed sedimentation coefficient ($S_{20,w} = 1.80$ Svedberg units). In the calculations $\bar{V}$ was taken as 0.731, the value calculated from the amino-

acid composition[12–14]. If the value of 0.751 used by other workers is substituted, the molecular weight is a little higher (18,600). It was not possible to distinguish more than one reasonably symmetrical peak in the high velocity experiments, but the rather rapid broadening of the peak by diffusion would have made it difficult to carry out a detailed analysis of the curve for evidence of heterogeneity. Certainly there was no significant trend of molecular weight with time in the low-speed experiment, which would be expected if small aggregates were present in significant proportions.

Electrophoresis

The haemoglobin of the sea lamprey (*P. planeri*) has shown two major haemoglobin components on starch gel electrophoresis[14–16]; the larval form of the lamprey also has two components, both of which are stated to be different from those in adults. On the other hand the Pacific lamprey (*Entosphenus tridentatus*) appears to have a single haemoglobin component on filter paper electrophoresis[17].

All 26 specimens of *L. fluviatilis* haemoglobin submitted to filter paper electrophoresis at pH 8.6 were found to have two major components and one small minor component. The latter was well shown after benzidine staining. The same components were observed on starch block electrophoresis and electrophoresis in agar at pH 6.5 (Fig. 1). Components with similar mobilities were seen in two specimens of haemo-

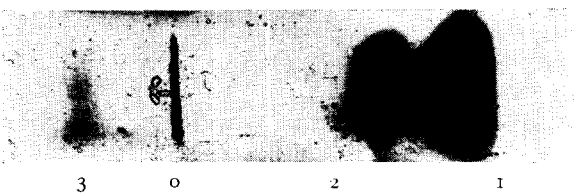

Fig. 1. Two major components (1 and 2) and a minor component (3) of the haemoglobin of *Lampetra* after electrophoresis in agar at pH 6.5. The origin is marked 0.

globin from the sea lamprey, *P. marinus*, submitted to filter paper electrophoresis. The possession of two major haemoglobin components would therefore appear to be the rule in all adult members of the genera *Lampetra* and *Petromyzon*, but not of *Entosphenus*. The isoelectric point of lamprey haemoglobin in phosphate buffer, ionic strength 0.05, is pI = 5.6–5.7, falling within the range of isoelectric points of invertebrate haemoglobins (pI = 4.5–6.0) as opposed to those of most higher vertebrates (pI = 6.7–7.2)[18,19].

Amino acid composition

The amino acid composition of lamprey haemoglobin, purified by starch block electrophoresis, is given in Table I. Amino-acid compositions of human myoglobin[20] and haemoglobin[21], and the haemoglobin of a teleost fish, *Thunnus thynnus*[22], are included for comparison. It will be noted that there are considerable differences in the proportions of several amino acids from human myoglobin and from the haemoglobins of higher vertebrates.

Attempts were made to determine the N-terminal amino acids of both lamprey haemoglobin components by the fluorodinitrobenzene method[23]. No N-terminal amino

acids were detected by this technique, which has previously been applied successfully to the haemoglobins of several other species. This result does not exclude the possibility that N-terminal proline is present, but suggests that the N-terminal amino groups may be blocked, *e.g.* by acetylation or cyclization.

TABLE I

AMINO ACID COMPOSITION OF LAMPREY HAEMOGLOBIN, HUMAN MYOGLOBIN AND THUNNUS AND HUMAN HAEMOGLOBINS

Composition in grams of amino acid/100 g protein.

Amino acid	*Lamprey Hb*	*Human Mb*	*Thunnus Hb*	*Human Hb*
Aspartic acid	9.3	8.3	11.2	9.6
Glutamic acid	7.2	16.2	6.7	6.6
Glycine	2.4	6.1	4.3	4.3
Alanine	10.3	5.6	8.5	9.2
Valine	8.2	3.4	7.3	10.4
Leucine	4.3	8.9	11.7	14.0
Isoleucine	4.4	3.4	5.7	< 0.02
Serine	8.3	4.4	4.5	4.1
Threonine	4.4	2.9	4.4	5.1
Methionine	2.9	1.5	2.8	1.3
Proline	4.6	4.8	2.4	5.0
Phenylalanine	8.3	4.3	6.4	7.3
Tyrosine	4.3	1.0	4.6	3.1
Histidine	1.0	12.9	4.1	8.3
Lysine	10.4	22.3	8.6	9.3
Arginine	2.9	2.5	8.6	2.8

Sulfhydryl groups

The number of sulfhydryl groups in lamprey carboxyhaemoglobin, both native and denatured, was determined by amperometric titration with phenyl mercuric hydroxide. The buffers used were borate ($Na_2B_4O_7$, 0.02 *M*; KCl, 0.15 *M*) and ammonia (NH_4Cl, 1.0 *M*; NH_4OH, 1.0 *M*). The protein was denatured by the addition of sodium dodecyl sulphate (approx. 400 moles/mole of protein). The native protein was found to have 1.0 SH per molecule (mol. wt. 17,000) and the denatured protein 1.9 SH per molecule. The same titres were obtained in both borate and ammonia buffers. Titrations of both the native and denatured protein were carried out in the presence and absence of sodium sulfite (0 to 0.1 *M*). The fact that the titres were independent of the sulfite concentration is evidence that there are no disulfide bonds in the molecule.

Absorption spectrum

The absorption spectrum of lamprey carboxyhaemoglobin has been examined in detail from 250–650 mμ. In the range 320–650 mμ the spectrum appears to be identical with that of human adult carboxyhaemoglobin (α band at 574 mμ, β at 540 mμ γ at 418 mμ and δ at 343 mμ, with the same extinction ratios). However, differences were apparent at shorter wavelengths. The ratio of predominantly protein absorption at 290 mμ to haem absorption at 343 mμ was 1.16 as compared with 0.93 in normal adult human carboxyhaemoglobin. Moreover, the tryptophan fine-structure band was less prominent than in other haemoglobins and at an unusually long wavelength

(291.2 mμ or longer). The major haemoglobin components were examined as separated zones in agar and showed no perceptible differences in this respect.

DISCUSSION

Lamprey haemoglobin consists entirely of units with molecular weight 17,200 each containing a single haem and, presumably, a single polypeptide chain. As expected of a pigment in which very little or no haem–haem interaction is possible, the oxygen equilibrium curve is hyperbolic[24]. In this respect lamprey haemoglobin resembles myoglobin, but the oxygen affinity of the latter is much greater under comparable conditions. Moreover, in its absorption spectrum and other properties lamprey haemoglobin resembles the haemoglobins rather than the myoglobins of higher vertebrates. Thus, there is a pronounced Bohr effect, which may be related to the presence of a free sulfhydryl group in the molecule; in other vertebrate haemoglobins the magnitude of the Bohr effect is related to the number of free sulfhydryls[25]. Myoglobin, which has an extremely small Bohr effect, has no sulfhydryl groups. The haemoglobin of the Californian hagfish, *Poliostrema stouti*, belonging to another order of cyclostomes, is reported to be hyperbolic and to have no Bohr effect[26]; it would be of interest to determine whether any thiols are present in the molecule. The presence of a sulfhydryl group in lamprey haemoglobin also shows that haemoglobins containing these groups do not necessarily aggregate into dimers or tetramers.

The presence of two electrophoretically separable major components in all adult *Lampetra* and *Petromyzon* studied, and of two other components in larval *P. planeri*[15,16], suggests that the four haemoglobins are synthesized under the control of four distinct genes. The universal presence of multiple haemoglobins occurs in the horse, the fowl and many other vertebrates[1]; this situation is quite distinct genetically from the haemoglobin polymorphisms found in man, monkeys, cattle, sheep and goats, in which different members of the same species show different components. In man at least three haemoglobin genes are assumed to be present controlling the synthesis of α, β and γ polypeptide chains; foetal and normal adult haemoglobins are produced by combination of these chains into $\alpha_2\gamma_2$ and $\alpha_2\beta_2$ units, respectively (see ref. 27).

A tentative picture of the evolution of the haemoglobin molecule can be drawn from available evidence. It would appear likely that the first pigment of this type, capable of reversible combination with oxygen, arose through attachment of haem —present in all free-living organisms— to a protein of molecular weight about 17,000. It is possible that at one stage haemoglobin in blood cells and myoglobin in tissue cells were identical. In any case from very early times they followed independent evolutions, myoglobin remaining as a storage pigment with a high oxygen affinity and haemoglobin becoming progressively adapted to fulfil its main function of oxygen transport in different species. Two important events occurred during the adaptation of the latter[28]. One was the acquisition of a Bohr effect, which facilitates oxygen liberation in the tissues. This may have arisen through incorporation of cysteine into the molecule, and represents the stage of development found in lampreys today. The second event was the aggregation of units of molecular weight about 17,000 to give the haemoglobins of approx. 68,000 found in all higher vertebrates. This would have permitted haem–haem interaction during oxygenation, with the development of a sigmoid oxygen equilibrium curve which is nicely adapted to carry out oxygen transport under a wide range of conditions in higher organisms.

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