HEMOGLOBIN COMPONENTS FROM TROUT (*SALMO IRIDEUS*): DETERMINATION OF THE CARBOXYL AND AMINO TERMINAL SEQUENCES AND THEIR FUNCTIONAL IMPLICATIONS

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Received 8 June 1973

1. Introduction

The blood of trout (*Salmo irideus*) contains several electrophoretically distinct hemoglobin components, as has often been reported for fish hemolysates [1, 2]. The isolated trout hemoglobin components proved to possess widely different functional properties [3].

The component with the lowest electrophoretic mobility at pH 8.5 (called Hb Trout I) is characterized by a cooperative O_2 binding curve (n = 2.5 from pH 6–9). The overall free energy of interaction for Trout I is similar to that characteristic of mammalian hemoglobins ($\Delta F_i \geqslant 2.5$ Kcal/mol) [4, 5]. In contrast to mammalian hemoglobins, the position of the ligand binding curve for Trout I is invariant with pH (from 6–9), invariant with the composition of the medium (in particular the presence of organic phosphates), and almost invariant with temperature (ΔH overall $\leqslant$ 1 Kcal/site).

The other major component of trout blood (called Hb Trout IV) has the greatest electrophoretic mobility at pH 8.5 and shows a very large pH dependence of the shape of the O_2 binding curve. The position of the O_2 binding curve has a large pH dependence and at low pH values (below 6.5) the molecule is only partially saturated in air. This property, which is characteristic of many fish hemoglobins, in known as the Root effect [6]. In the blood of trout this seems to be an exclusive property of Hb Trout IV [3]. The O_2 binding properties of this component are influenced by organic phosphates as well as by temperature.

Knowledge of the functional properties of the various components has suggested different physiological roles for the different hemoglobin components [7]. It appears possible to rationalize the multiplicity of components in terms of physiological requirements of teleost fishes, where hemoglobins may play the double role of providing O_2 to the tissues and to the swim bladder [8].

The molecular and functional properties of the isolated components, including kinetic aspects of the reaction with ligands, have been extensively investigated [7 and data to be published]. However, understanding of the functional properties in structural terms demands knowledge of the structural features of the molecules in question, and in particular their chemical properties. Work directed towards the determination of the primary structure of the main components from the blood of trout has therefore been undertaken. This note reports preliminary information dealing with the determination of the carboxyl and amino terminal sequences of Hb Trout I and Hb Trout IV as well as some information on Hb Trout II. The relevance of our findings for the interpretation of the functional properties is briefly discussed.

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Table 1
Amino acids released (equivalents per $\alpha\beta$ dimer) from Hb Trout components after digestion with carboxypeptidase A or B.

	Carboxypeptidase A	Carboxypeptidase B**
Trout I	Tyr (1.0), Phe (1.0)	Tyr (2.0) Phe (1.0) Arg (2.0) Lys (1.0) Ala (0.7)
Trout II	Tyr (1.0), Phe (1.0)	Arg (2.0) Phe (1.0) Tyr (2.0) Lys (1.0)
Trout IV	His (1.0), Tyr (1.0) Gln (0.5)	Arg (0.7), Tyr (0.4)

** The release of non-basic amino-acids by CPB may be connected with the presence of SDS, with the time of digestion (2 hr) and with the enzyme/substrate ratio (1/50 w/w).

2. Materials and methods

Preparation of hemoglobin and purification of the various components was performed as previously reported [3]. From the homogeneous components, globins were prepared by the acid–acetone procedure [5]. Globins obtained by the standard method, however, are insoluble at neutral pH in water or dilute buffer, and therefore are probably denatured.

Starch gel electrophoresis in 8 M urea and mercaptoethanol was performed according to standard procedures [9]. Digestions with Worthington, DFP-treated carboxypeptidase A (CPA) and B (CPB) were carried out according to Ambler [10], using 0.05 M Tris–HCl buffer, pH 8.0, in the presence of 3–15 mM sodium dodecyl sulfate (SDS)*. Samples were withdrawn at the appropriate time intervals and quenched with trichloroacetic acid. The amino acid content was determined with a Bio Cal BC 200 or an Optica amino acid analyzer.

The *N*-terminal amino acids were identified by the modified dansylation technique for proteins suggested by Gray [12]. The DNS-amino acids were identified by the thin layer chromatographic method described by Woods and Wang [13].

Manual determination of the *N*-terminal sequence of the chains was carried out with dansyl chloride according to the rapid degradation procedure described by Gray [12].

3. Results

Each of the two main components of trout blood (namely Hb Trout I and Hb Trout IV) is composed of two different types of chains, as shown by starch gel electrophoresis in 8 M urea and mercaptoethanol. As discussed below, these two types of chains may be designated as α-like and β-like on the basis of their *C*-terminal sequences, and since these two hemoglobin components do not possess common chains, they are designated as $\alpha_2^I\beta_2^I$, $\alpha_2^{IV}\beta_2^{IV}$.

Isolation of the chains was attempted by chromatography on CM-cellulose in 8 M urea and 0.05 M mercaptoethanol. By properly modifying the procedure employed for human hemoglobin [14], in a manner to be reported in detail, it was possible to obtain pure chains from the globin of Trout IV. Chromatographic fractions were assigned to a certain type of chain on the basis of the electrophoretic mobility and, more rigorously, on the basis of the *C*-terminal amino acid sequence (see later). Similar separation procedures when applied to Hb Trout I (or Trout II) proved to be less successful, and only one chain could be obtained in reasonably pure form.

Carboxyl terminal sequences were obtained by carboxypeptidase digestion of the hemoglobins in SDS. Table 1 shows the type and number of amino acid residues released per $\alpha\beta$ unit after digestion with either CPA or CPB. Digestion of the protein with CPA always released: His and Tyr (1 equivalent per $\alpha\beta$ dimer) from Hb Trout IV, and Phe and Tyr (1 equivalent per $\alpha\beta$ dimer) from Hb Trout I (and Hb Trout II). On the other hand, digestion with CPB released arginine from all three components, along with other amino acid residues, including tyrosine.

These results are somewhat at variance with those obtained by Bulher on hemoglobin from *Salmo gairdneri* [15], for which no Arg was released by CPB addition.

The *C*-terminal sequences were deduced from the analysis of: i) the time dependence of the digestion with either CPA or CPB; ii) the digestion of the purified chains in the case of Hb trout IV. For Hb Trout I this second approach could be used only on partially purified chains.

* In the absence of SDS, Hb Trout I is not susceptible to the action of either CPA or CPB, while removal of residues has been obtained with Hb Trout IV in the native state [11].

Table 2
Summary of *N*- and *C*-terminal sequences of various hemoglobins.

Man	α	Val-Leu-Ser-Pro-Ala-Asp-	-Leu-Thr-Ser-Lys-Tyr-Arg
	β	Val-His-Leu-Thr-Pro-Glu-	-Leu-Ala-His-Lys-Tyr-His
Trout I	αβ	Val-	α -Lys-Tyr-Arg β -Gly-Ser-Arg-Tyr-Phe
Trout IV	α	Val-X-Ser-Ala	(Leu, Glu, Ala)-Lys-Tyr-Arg
	β	Val-Asx-X-Thr-Asx	(Val, Leu, Ala) Arg-Gln-Tyr-His
Catostomus Clarkii (17)	α	Acetyl-Ser-Leu-Ser-Asp-	Glu-Lys-Tyr-Arg
	β Cathodal	Val-Glu-Trp-Ser-Asp	Tyr,Phe
	β Anodal	Val-Gly-Trp-Thr-Asp	Arg-Gln-Tyr-His

Table 2 shows the amino acid sequences of the *C*-terminal region on the trout hemoglobin components. The sequence –Tyr–Phe for Hb Trout I, which is the likely one on the basis of homologies, is confirmed by: i) partial digestion of Hb Trout I in the absence of SDS with high CPA concentration; ii) sequencing of a peptide obtained by CNBr fragmentation of Hb Trout I.

The dansylation method for identification of *N*-terminal residues was carried out on the purified hemoglobins as well as on some isolated chains. The only residue found at the *N*-terminal position was valine for all three hemoglobin components. It was impossible, however, to decide whether both the α and β chains of Hb Trout I and II have freely accessible α-amino groups or whether one of the chains has a blocked amino group, as reported for other fishes [16, 17]. For Hb Trout IV neither α nor β chains have blocked *N*-termini. Partial *N*-terminal sequences of both the α and β chains of Hb Trout IV, obtained by the rapid degradation procedure [12], are reported in table 2.

4. Conclusion

Consideration of the data reported above, in conjunction with the knowledge of the functional properties of the various hemoglobin components from trout, allows us to draw the following conclusions:
a) The *C*-terminal sequence of the α chains in trout is less variable than that of the β chains, in agreement with extensive observations already available. In all cases tyrosine seems to be present in the penultimate position. This is in agreement with the stereochemical model of hemoglobin function, based largely on X-ray crystallographic studies of human hemoglobin [18]. The stereochemical model assigns an essential role to this residue in the functional properties of the molecule, and in particular for the cooperative effects observed in O_2 binding.
b) In Hb Trout I the absence of Bohr effect, and possibly of other heterotropic phenomena, may be correlated with the substitution of Phe for the *C*-terminal His of the β chains. The presence of a hemoglobin component containing Phe in the terminal position of the β chains may be common in fast swimming fishes [see 17]. The lack of a Bohr effect may have definite physiological significance in these fish. Our data on Hb Trout I and II appear remarkably consistent with the results of Powers and Edmundson [17] on *Catostomus clarkii.*
c) On the other hand, in Hb Trout IV, the presence of marked heterotropic effects is associated with the presence of His at the *C*-terminal position of the β chain. Although the Root effect cannot be ascribed exclusively to the *C*-terminal His, the participation of this residue in the pH dependent effects is shown by the functional modifications observed after digestion of the native protein with CPA [11].
d) As shown by X-ray crystallographic studies of human hemoglobin [18, 19], the binding of organic phosphates and notably of 2-3, diphosphoglycerate (DPG), involves interaction of the allosteric effector

with the following residues: Val 1 (β) (the α-NH_2 group), His 2 (β), Lys 82 (β), and His 143 (β). As is clear from table 2, two of these four 'binding sites' i.e. His 2 and His 143 (β) appear to be missing in Hb Trout IV. Addition of ATP, the physiologically important phosphate in fish, to Hb Trout IV decreases the oxygen affinity only about half as much as in human hemoglobin [20] and, in general, the effect of organic phosphates on the O_2 binding of Hb Trout IV is considerably smaller than that observed with human hemoglobin [5, 21]. Similar correlations were pointed out by Powers and Edmundson [17] in the case of *Cs. clarkii.* The beautiful correlation between structural and functional data is independent evidence for the role of precise charge complementarity in regulating the interactions between hemoglobin and organic phosphates.

Acknowledgements

We wish to thank Mr. M. Coletta for hard and skillful work involved in the chains separation, Drs. C Bonaventura and B. Giardina for stimulating discussions and Professor E. Antonini and Professor P. Fasella for their continuous interest in the work.

References

[1] Riggs, A. (1970) in: Properties of Fish Hemoglobins. Fish Physiology 4, 209.
[2] Giovenco, S., Binotti, I., Brunori, M. and Antonini, E. (1970) Intl. J. Biochem. 1, 57.
[3] Binotti, I., Giovenco, S., Giardina, B., Antonini, E., Brunori, M. and Wyman, J. (1971) Arch. Biochem. Biophys. 142, 274.
[4] Wyman, J. (1964) Advan. Protein Chem. 19, 223.
[5] Antonini, E. and Brunori, M. (1971) in: Hemoglobin and Myoglobin in their reactions with ligands. North-Holland Research Monographs Frontiers of Biology 21.
[6] Root, R.W. (1931) Biol. Bull 61, 427.
[7] Brunori, M., Giardina, B., Bonaventura, J., Barra, D. and Antonini, E. (1972) Intern. Symp. Comp. Physiol., in press.
[8] Fange, R. (1966) Physiol. Rev. 46, 299.
[9] Chernoff, A.I. and Pettit, N.M. (1964) Jr. Blood 24, 750.
[10] Ambler, R.P. (1967) Methods in Enzymology 11, 436.
[11] Brunori, M., Bonaventura, J., Bonaventura, C., Giardina, B., Bossa, F. and Antonini, E. (1973) Mol. Cell. Biochem., 1, 189.
[12] Gray, W.R. (1972) Methods in Enzymology 25, 121, 333.
[13] Woods, K.R. and Wang, K.T. (1967) Biochim. Biophys. Acta 133, 369.
[14] Clegg, J.B , Naughton, N.A. and Weatherall, D.J. (1966) J. Mol. Biol. 19, 91.
[15] Buhler, D.R. (1963) J. Biol. Chem. 238, 1665.
[16] Hilse, K. and Braunitzer, G. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 433.
[17] Powers, D.A. and Edmundson, A.B. (1972) J. Biol. Chem. 247, 6686.
[18] Perutz, M.F. (1970) Nature 228, 726.
[19] Arnone, A. (1972) Nature 237, 146.
[20] Brunori, M., Giardina, B., Chiancone, E., Spagnuolo, C., Binotti, I. and Antonini, E. (1973) European J. Biochem., in press.
[21] Benesch, R. and Benesch, R.E. (1969) Nature 221, 618.