High-performance liquid chromatographic separation and partial characterization of the α- and β-chains of Alaskan sockeye salmon hemoglobin

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ABSTRACT

The life cycle of the sockeye salmon (*Oncorhynchus nerka*) includes both fresh water early development and oceanic adult periods. These salmon have multiple hemoglobins whose functional and evolutionary significance is still unresolved. The presence of these various hemoglobins may be advantageous to assuring adequate oxygen transport over a wide variety of environmental conditions. Functional differences between hemoglobin components in oxygen affinity, thermal stability and autoxidation have been reported. In order to better understand the molecular basis of these functional properties of the hemoglobin components, we have identified and characterized several of the polypeptide chains of sockeye salmon hemoglobins. The hemoglobin components were separated by chromatography into cathodal and anodal components. These components were then resolved by reversed-phase high-performance liquid chromatography. A gradient system was developed which could separate seven polypeptide chains. Amino acid compositions and sequence analysis of the polypeptide chains allowed the classification of some of the polypeptide chains as either α- or β-chains.

INTRODUCTION

Over the past decade increased studies of the physical and chemical properties of fish hemoglobins have contributed to a greater understanding of the variability of the structure and function relationships exhibited by these unique oxygen transport proteins as compared to mammalian hemoglobins. Comparative aspects of the structure and function of these proteins have received much attention[1–3]. Fish are unique in that many species possess a large number of hemoglobin components whose functional activity may be altered by a variety of environmental or physiological factors.

The study of many diverse species of fish have demonstrated that electrophoretically distinct hemoglobin components of individual fish species usually exhibit different oxygen-binding characteristics[4,5]. In addition, recent comparative studies on the thermostability of fish hemoglobins reveal lower resistance to thermal denaturation and oxidation[6,7].

Reversed-phase high-performance liquid chromatography (HPLC) was applied to the separation of human hemoglobin chains[8] and hemoglobin chain variants[9–11]. These methods with modifications have been extended to primates[12,13], other mammals[14–19] and reptiles[20]. In studies of fish hemoglobin, reports of HPLC analysis of the individual α- and β-chains have become more common in the literature. In studies on bluefin tuna, a 0.1 M ammonium acetate–formic acid buffer with acetonitrile gradient was used[21], while in Goosefish, with only one component, isocratic elution with a mobile phase of 0.8 M sodium perchlorate–methanol–acetonitrile–nonylamine–phosphoric acid (29:5:66:0.1:0.5, v/v) was successful[7]. The single hemoglobin of the Crucian was separated into its chains by using a potassium phosphate (pH 2.5)–acetonitrile–methanol system[22]. In order to study developmental changes during smoltification and aging of fish in a system not prone to produce the artifacts that can accompany electrophoresis, we have worked on developing an HPLC procedure for separating the hemoglobin chains associated with the multi-component systems of fish.

EXPERIMENTAL

Chemicals

HPLC-grade water and acetonitrile were purchased from Fisher (Springfield, NJ, U.S.A.) and trifloroacetic acid (TFA) was sequential grade (Pierce, Rockford, IL, U.S.A.). All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Apparatus

Ion-exchange chromatography was performed on a Waters 650 fast protein liquid chromatographic (FPLC) system, equipped with a Model 484 variable-absorbance detector and a Spectrophysics Model 9200 integrator. A Waters HPLC system with a Model 441 fixed-wavelength detector and Baseline (Dynamic Solutions, CA, U.S.A.) data processing software was used for the chain separation.

Hemoglobin preparation

Blood samples were obtained from sockeye salmon, *Oncorhynchus nerka*, caught in the Kenai River, Alaska. Individual fish were bled from the caudal vein, and the blood was transferred to flasks containing cold 1% sodium chloride. Red cell suspensions were centrifuged at 4°C, and the supernatant was discarded. Packed red cells were washed at least three times with the above saline solution. After the final wash, the red cells were osmotically lysed by the addition of two volumes of cold deionized distilled water. Cellular debris was removed by centrifugation at low speed for 10 min at 4°C, followed by centrifugation of the hemoglobin supernatant for 30 min at 8160 g.

Preparation of globins for HPLC analysis

Globin was precipitated from the hemoglobins at -20°C in acidified acetone.

TABLE I

SUMMARY OF HPLC GRADIENT CONDITIONS FOR SEPARATION OF SALMON HEMO-GLOBIN CHAINS

Eluent A = 20% acetonitrile containing 0.1% TFA; eluent B = 80% acetonitrile containing 0.1% TFA. Column: Vydac C_4, 250 mm × 4.6 mm I.D. (300 A).

Event No.	Time (min)	Flow-rate (ml/min)	Composition (%)	
			Eluent A	Eluent B
1	0.0	1.0	100.0	0.0
2	10.0	1.0	99.0	1.0
3	15.0	1.0	60.0	40.0
4	75.0	1.0	75.0	65.0
5	80.0	1.0	0.0	100.0
6	89.0	1.0	100.0	0.0
7	90.0	0.0	100.0	0.0

Hemoglobin α- and β-chains were isolated by reversed-phase HPLC of a solution with 8 M urea and 1% mercaptoethanol (pH 3). When using relatively large amounts of hemolysate, we found that urea treatment insures complete dissociation of the hemoglobin molecule. Chains were separated on a Vydac C_4 column using an aceto-nitrile gradient with 0.1% TFA as a counter-ion at pH 2.1. The flow-rate was 1 ml/min. Peaks were detected by absorbance at either 214 or 254 nm. Table I summa-rizes the gradient.

Amino acid analysis

Samples were hydrolyzed in constant-boiling hydrochloric acid (Pierce, Rock-ford, IL, U.S.A.) with 0.1% phenol at 108°C for 24 h and analyzed in a Beckman 6300 analyzer[13].

Protein sequence analysis

Hemoglobin and peptide samples were used for N-terminal sequence analysis on an Applied Biosystems Model 470A gas phase protein sequencer[13]

Isolation of hemoglobin component fractions

Isolation of the anodal and cathodal fractions was carried out by ion-exchange chromatography on DE-52 (diethylaminoethyl cellulose) developed with 0.05 M Tris–HCl buffer (pH 8.4). Individual fractions and components were eluted from the column with 0.5 M sodium chloride in the same buffer. This procedure was modified by using the Waters advanced protein chromatography system with either a Waters Protein-Pak DEAE column or a Waters Accell QMA column.

RESULTS AND DISCUSSION

The uniqueness and complexity of fish hemoglobins compared to mammalian hemoglobins are well demonstrated by the extensive hemoglobin polymorphism ob-

served in the various species of trout and salmon[23-25]. These electrophoretically distinct hemoglobins appear to provide an adaptive mechanism necesary for the life cycle of these fish. Each species of salmon exhibits a similar life cycle in many respects, with spawning and early development occurring in fresh water, giving way to the highly saline environment of the ocean as adults. An increase in the number of hemoglobin components has been reported to occur during this development[25]. Also differences in thermal stability of the anodal and cathodal hemoglobin components of salmon hemoglobins have been observed[2,5]. Since sockeye salmon can show five to thirteen hemoglobin components[6,26], the chain compositions of the sockeye salmon hemoglobins were analyzed by reversed-phase HPLC.

Fig. 1 shows a representative elution profile of hemoglobin chains from the total salmon hemoglobin preparation. Good separation of the chains was obtained with the Vydac C_4 reversed-phase column within 65 min. Between time 45 to 60 min, seven major peaks and two minor peaks could be resolved. In an attempt to correlate the chains seen in Fig. 1 with the individual hemoglobin components which had been defined by electrophoretic mobility[6,26], individual hemoglobin components which had been partially separated by ion-exchange chromatography on DE-52 (Whatman) were analyzed by HPLC. HPLC analysis of an overlapping triplet of peaks easily demonstrated that the middle component contained hemoglobin chains associated with its neighboring components[5]. The HPLC profile of the first-eluting hemoglobin component of this triplet, in which only single α- and β-chains are seen, is illustrated in Fig. 2. The analysis by this HPLC method of homogeneous fractions separated by either ion-exchange chromatography or by gel electrophoresis will allow us to confirm the hemoglobin chain number and designations proposed by Tsuyuki and Ronald[26].

Preliminary amino acid analysis of the globin peaks in Fig. 2 led to the identification of an α-chain (Table II). Since the α-chains of salmonoid fish usually have
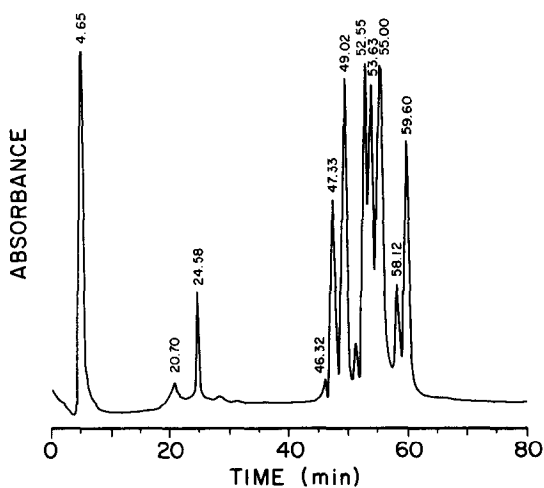

Fig. 1. HPLC separation of the sockeye salmon hemoglobin chains (200 μg) on a Vydac C_4 column (250 mm $\times$ 4.6 mm I.D.) in a 0.1% TFA–acetonitrile system. The gradient was from 20 to 80% acetonitrile with detection at 214 nm (see Table I).
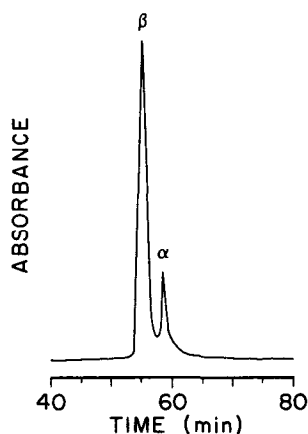
Fig. 2. HPLC separation of the sockeye salmon hemoglobin chains from a column fraction previously eluted from DE-52 cellulose. Elution conditions were the same as in Fig. 1.

TABLE II

COMPARISON OF SALMONOID FISH α-CHAIN AMINO ACID COMPOSITION WITH SOCK-EYE HEMOGLOBIN HPLC PEAK

Amino acid	Trout I[a]	Trout IV[a]	Carp[a]	Atlantic[a] salmon I	Sockeye[b] salmon C1
Asx	13	15	14	14	13.8
Thr	6	6	3	6	7.6
Ser	11	9	11	9	9.1
Glx	3	9	6	4	6.9
Pro	6	7	8	6	5.8
Gly	14	6	11	14	12.1
Ala	18	16	17	20	20.4
Val	12	10	12	12	13.2
Met	4	3	4	4	3.4
Ile	8	10	8	6	6.9
Leu	13	16	14	15	13.3
Tyr	3	4	4	3	3.4
Phe	7	6	6	7	7.3
Lys	13	13	14	11	11.8
His	6	6	5	5	3.9
Arg	3	3	3	4	3.2
Trp	2	2	2	2	N.D.[c]
Cys	—	1	—		N.D.[c]

[a] References: trout, ref. 23; Atlantic salmon, ref. 27.
[b] Based on a 142 amino acid composition; C1 designation for this component is based on ion-exchange chromatography and electrophoresis[5].
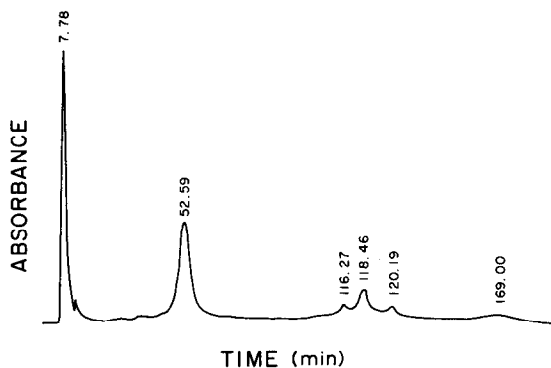[c] N.D., not determined.

Fig. 3. Component separation of the hemolyzate of the sockeye salmon by ion-exchange chromatography (200 μl). Column: Accell QMA (10 mm × 8 mm I.D.); buffer A, 10 mM sodium phosphate, pH 8.0; buffer B, buffer A + 1 M sodium chloride. Linear gradient from 1 to 100% B over 200 min. Flow-rate, 0.5 ml/min, detection at 415 nm. Numbers at peaks indicate retention times in min.

acetylated amino terminals, protein sequencing could not be used to identify chain type. However, the amino acid analysis clearly shows that the sockeye salmon hemoglobin component has an α-chain which is homologous to Type I α-chain of Atlantic salmon and trout[20,27], as can be seen in he glycine and valine compositions. Separation and isolation of the globin chains by HPLC has allowed for a partial determination of the amino acid sequences of the β-chains for these components[28]. So far there is 90–100% homology between trout hemoglobin β-chains and sockeye hemoglobin β-chains depending on which hemoglobin components are compared[28]. The HPLC separation of the chains now allows us to see whether all the heterogeneity of the components seen in electrophoresis and isoelectric focusing is directly related to polypeptide gene products or to post-translational events such as non-enzymatic glycosylation.

In an attempt to purify sockeye salmon hemoglobin components for HPLC analysis, we tried ion-exchange chromatography using a Waters Protein-Pak DEAE column. We found that this column, used under conditions identical to those previously used with the DE-52 column[5], bound very little of the hemoglobin components. This may be because an initial pH of 8.4 is not high enough for sockeye salmon hemoglobin binding to this particular column. When we used an Accell QMA column with a sodium phosphate–sodium chloride system, we were able to separate hemoglobin into several fractions. Fig. 3 shows a typical separation. The acidic fraction is eluted first, followed by a more basic fraction. HPLC analysis can be used to identify the homogeneity of these fractions based upon their chain compositions.

Since hemoglobin components change around the time of smoltification[25], the use of this HPLC procedure will allow us to quantitate the amounts of various chains synthesized during this transitional period. It will also allow us to monitor post-translational changes such as glycosylation which may occur during salmon spawning. The use of HPLC analysis of individual fish hemoglobins will obviate the autoxidation artifacts commonly seen in electrophoresis of stored fish hemoglobin samples. The reproducibility and sensitivity associated with HPLC analysis improve the use of the hemoglobin molecule as a monitor of early development in fish.

ACKNOWLEDGEMENTS

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