PHYSICOCHEMICAL PROPERTIES OF FISH HEMOGLOBINS ISOLATED FROM GULF OF MEXICO SPECIES

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Abstract—1. Vertical starch-gel electrophoresis at pH 8.6 revealed specific hemoglobin multiplicity in most hemoglobin hemolysates isolated from 13 Gulf of Mexico fish species.
2. All hemoglobins isolated are tetramers based upon gel filtration chromatography.
3. Isothermal denaturation in the presence of urea indicates large differences in the denaturation and unfolding behavior at 25°C.
4. Significant Root effects were observed below pH 7.0 for individual hemoglobin components of menhaden, Brevoortia patronus, and the striped bass, Morone saxatilis.
5. Large differences in the rate of autoxidation were determined for many of the hemoglobins examined.
6. Two species of catfish, Arius felis and Bagre marinus, possessed the most stable hemoglobins and lowest rate of autoxidation compared to the other species of fish studied.

INTRODUCTION

Studies of physical and chemical properties of fish hemoglobins during the past decade have provided comparative biochemists with a wide range of opportunities to elucidate the vital relationships between structure and functional behavior of these oxygen transport proteins for many species of fish. The observed uniqueness and complexity of fish hemoglobins compared to mammalian hemoglobins is well documented by the presence of multiple hemoglobins which can be separated and distinguished electrophoretically (Riggs, 1970; Binotti et al., 1971; Giles and Vanstone, 1976; Harrington, 1983). This expression of hemoglobin polymorphism is linked to the necessary functional characteristics of these hemoglobins required by these species of fish for either adaptive purposes or survival within their environmental habitats.

Over the past several years within our laboratory, we have been investigating the structure–function relationship of polymorphic hemoglobins isolated from several species of North Pacific salmon, the pink salmon, Oncorhynchus gorbuscha, the coho salmon, Oncorhynchus kisutch, and the sockeye salmon, Oncorhynchus nerka. These comparative studies initially focused upon the physical properties of these hemoglobins as related to their resistance to thermal denaturation (Harrington, 1982, 1983, 1985). Our more recent studies have focused upon the oxygen binding properties of the king salmon, Oncorhynchus tshawytscha, (Harrington, 1986) and the sockeye salmon, Oncorhynchus nerka (Sauer and Harrington, 1988).

We have extended these studies of multiple fish hemoglobins to several diverse species of fish found in the sub-tropical waters of the Gulf of Mexico. Included in this investigation are the hemoglobins isolated from the pearly razorfish, Hemipteronotus novacula, short big eye, Pristigenys alta, red poregy, Pogrus sediticm, hardhead catfish, Arius felis, jack cravelle, Caranx hippos, menhaden, Brevoortia patronus, and the striped bass, Morone saxatilis.

Materials and Methods

Hemoglobin preparation

Blood samples were collected during several cruises of the R/V Verrill (Dauphin Island Sea Laboratory, Dauphin Island, Alabama). Fish were caught at depths varying from 6 m to 100 m. Individual fish were bled from the caudal vein and the blood was transferred to flasks containing cold 1% NaCl to which heparin was added. All blood samples were filtered through gauze prior to centrifugation at 4°C. Packed erythrocytes 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 2 vols of cold deionized/distilled water. Lysed membranes were removed by centrifugation at low speed for 10 min at 4°C, followed by centrifugation of the hemoglobin supernatant for 30 min at 10,000 rev/min. This last centrifugation was repeated twice.

All hemoglobin samples were equilibrated to pH 8.4 (0.05 M Tris–HCl) by passage through a Sephadex G-25 column. Separation and isolation of anodal and cathodal hemoglobin components were carried out by ion-exchange chromatography using DE-52 (diethyl aminoethyl cellulose, Whatman) developed with a 0.05 M Tris–HCl, pH 8.4.
Individual components were eluted from separable Kontes columns with a high ionic strength solution (0.5 M NaCl) in the same buffer. Several dilute components were concentrated by centrifugation using Amicon microcentrifuge centrifuge tubes prior to experiments and for storage under liquid nitrogen.

Electrophoresis

Identification of all hemoglobin components was carried out by vertical starch-gel electrophoresis at 4°C using a Tris–EDTA–borate buffer, pH 8.6, according to Smithies (1959). All electrophoreses were carried out at 240 V for 17 hr. Gels were stained with o-toluidine. Normal adult hemoglobin A was used as a reference marker for all electrophoretic runs.

Gel filtration chromatography

Gel filtration chromatography was carried out using a Sephadex G-100 column (60 x 1 cm) eluted with 0.1 M potassium phosphate, pH 7.3. All individual hemoglobin components were run as a 1:1 mixture with normal adult hemoglobin A at 4°C. Individual fractions (1.5 ml) were monitored at 540 nm.

Isothermal denaturation

Urea solutions of 1–9.5 M concentration were prepared by volumetric dilution using a 10 M stock solution. For absorbance determinations hemoglobin solutions were prepared by dilution in 5 ml volumetric flasks from the appropriate stock solution of fish hemoglobin component. All protein solutions contained 0.1 M potassium phosphate, pH 7.3. All absorption spectra in the Soret region (450–350 nm) were carried out on a Varian 2290 u.v./Visible recording spectrophotometer equipped with a temperature controlled compartment cell holder. Spectral measurements of each solution were recorded after 2 hr of equilibration at 25°C. The unfolding of each hemoglobin protein as a function of increasing urea concentration was followed by changes in absorbance at 413 nm, assuming a molar extinction coefficient of 1.25 x 10^5 (DiIorio, 1981). Protein concentrations varied between 10^-5 and 10^-6 M in heme (Harrington, 1986).

Root effect studies

The specific concentrated fish hemoglobin components to be analyzed were diluted within the cuvette containing the appropriate buffer to a hemoglobin (tetramer) concentration of 12–15 μM. The pH range examined varied from 8.5–5.4. Direct absorbance spectra were recorded from 700–500 nm region at cycle times of 10–45 min. To insure complete oxidation at the end of each series of spectral determinations, a small amount of potassium ferricyanide was added to each sample and the spectrum recorded. The kinetics of these oxidation reactions is presented as a plot of log % oxyhemoglobin remaining versus time.

Autoxidation

Hemoglobin samples were prepared from stock solutions with dilution into the appropriate buffer to a final hemoglobin concentration of between 12 and 15 μM (tetramer). Continuous spectra were recorded over the 700–500 nm region at cycle times of 10–45 min. To insure complete oxidation at the end of each series of spectral determinations, a small amount of potassium ferricyanide was added to each sample and the spectrum recorded. The kinetics of these oxidation reactions is presented as a plot of log % oxyhemoglobin remaining versus time.

RESULTS

The hemoglobin patterns evident by vertical starch-gel electrophoresis at pH 8.6 of total hemolysates from a variety of Gulf of Mexico fishes are shown in a composite diagram in Fig. 1. Extensive hemoglobin polymorphism was found for the red porgy, Pagrus sedecim, and Atlantic croaker, Micropogon undaltus. Each of the other total hemolysates are separable into either two major hemoglobin components or a major and minor component. All samples examined including the hemoglobins of the spot, Leiotomus xanthus, striped bass, Morone saxatilis, and gafftopsail catfish, Bagre marinus, indicated the presence of anodal components at pH 8.6. The Atlantic croaker contains at least two minor hemoglobin components that have high mobility in the cathodal direction.

Two exceptions to the normal hemoglobin polymorphism exhibited by most fish were found amongst the species examined in this study. Only a single hemoglobin component was evident electrophoretically for the hardhead catfish, Arius felis, and the gafftopsail catfish, Bagre marinus. The presence of a single hemoglobin in these catfish is similar to that observed for the spot fish, Leiotomus xanthus.

Separation of these electrophoretically distinct hemoglobin components within each species of fish was carried out using ion-exchange chromatography (DE-52) at pH 8.4. Resolution and isolation of each of the components were reproducible for different preparations. After isolation of each hemoglobin component, a determination of their approximate mol. wt was made by mixing (1:1) each hemoglobin component with normal adult human hemoglobin A and evaluating the chromatogram following elution off a Sephadex G-100 column. All the individual fish hemoglobins are tetrameric (mol. wt = 64,000) as evident by their co-elution with hemoglobin A, as well as the symmetry of each of these peaks. An example of this is presented in Fig. 2 showing the co-elution of the striped bass, Morone saxatilis, hemoglobin with hemoglobin A.

The effects of isothermal denaturation by urea were analyzed for many of these fish hemoglobins. Figs 3A and 3B present some of the spectrophotometric
results of the hemoglobins from the striped bass, *Morone saxatilis*, and the gafftopsail catfish, *Bagre marinus*, in the presence of urea. The denaturation transitions as a function of urea concentration were obtained as evident in Figs 4A and 4B. Interestingly, in each set of transitions, the catfish hemoglobins (Fig. 4A, gafftopsail; Fig. 4B, hardhead) exhibit significantly increased denaturation midpoints (D1/2) compared to the other hemoglobins examined. These profiles also indicate different degrees of transition steepness which is a reflection of the extent of cooperativity in the unfolding process of these hemoglobins. Table 1 presents a complete list of all the denaturation midpoints obtained from analysis of these denaturation profiles.

Susceptibility to autoxidation is rather common with fish hemoglobin systems. This is particularly evident at pH values of 7.0 or below. Figure 5 shows

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**Fig. 2.** Gel filtration chromatogram of a mixture (1:1) of striped bass hemoglobin and human hemoglobin A. Sephadex G-100, 0.1 M potassium phosphate, pH 7.3, T = 4°C.

**Fig. 3.** Absorbance spectra in the Soret region (450–350 nm) for (A) gafftopsail catfish in different concentrations of urea: (a) native protein, (b) 2 M urea, (c) 5 M urea, (d) 5.5 M urea, (e) 6 M urea, (f) 7 M urea, (g) 8 M urea; and (B) striped bass: (a) native protein, (b) 2 M urea, (c) 3 M urea, (d) 4 M urea, (e) 5 M urea, (f) 7 M urea, and (g) 8 M urea. All solutions contained 0.1 M potassium phosphate, pH 7.3, T = 25°C.

**Fig. 4.** The denaturation of several fish hemoglobin components by urea followed by changes in absorbance at 413 nm Soret band. (A) ○ menhaden (anodal component), ■ striped bass, and ▲ gafftopsail catfish and (B) ■ hardhead catfish, ▲ spot, and ○ ground mullet (cathodal component). All solutions contained 0.1 M potassium phosphate, pH 7.3, T = 25°C.
Table 1. Denaturation midpoints, D1/2, in the presence of urea for fish hemoglobins at T = 25°C

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>D1/2 (urea)</th>
<th>D2/2 (urea)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menhaden (anodal)</td>
<td>3.4 M</td>
<td>5.1 M</td>
</tr>
<tr>
<td>Menhaden (cathodal)</td>
<td>2.8 M</td>
<td>2.7 M</td>
</tr>
<tr>
<td>Ground mullet (anodal)</td>
<td>4.0 M</td>
<td>2.7 M</td>
</tr>
<tr>
<td>Ground mullet (cathodal)</td>
<td>3.8 M</td>
<td>3.5 M</td>
</tr>
<tr>
<td>Atlantic croaker (anodal)</td>
<td>3.5 M</td>
<td>3.6 M</td>
</tr>
<tr>
<td>Atlantic croaker (cathodal)</td>
<td>4.0 M</td>
<td>4.1 M</td>
</tr>
<tr>
<td>Catfish, hardhead</td>
<td>5.8 M</td>
<td>6.5 M</td>
</tr>
<tr>
<td>Catfish, gafftopsail</td>
<td>6.5 M*</td>
<td>6.5 M*</td>
</tr>
<tr>
<td>King salmon (anodal)</td>
<td>3.3 M</td>
<td>3.6 M</td>
</tr>
<tr>
<td>King salmon (cathodal)</td>
<td>4.1 M</td>
<td>5.3 M</td>
</tr>
<tr>
<td>Sockeye salmon (anodal)</td>
<td>3.6 M†</td>
<td>6.0 M†</td>
</tr>
<tr>
<td>Human hemoglobin A</td>
<td>6.5 M†</td>
<td>6.5 M†</td>
</tr>
</tbody>
</table>

*Harrington, 1986.
†Sauer and Harrington, 1988.
‡Elbaum et al., 1974.

the effect of hydrogen ion concentration on several hemoglobin components in the pH 6.3–6.5 range at 25°C in 0.1 M potassium phosphate buffer. Within 150 min at pH 6.5, autoxidation leads to 62% and 93% methemoglobin for the anodal and cathodal fractions of the ground mullet, *Menticirrhus americanus*, respectively. These rapid rates of methemoglobin formation stand in marked contrast to the extremely low rate of autoxidation for the hardhead catfish, *Arius felis* hemoglobin where only 5% methemoglobin is observed at pH 6.5.

On the functional side of this study we have examined the presence of possible Root effect behavior in several of these fish hemoglobins (Root, 1931). Figure 6 clearly demonstrates discrete oxygen saturation properties as a function of pH at 25°C. The anodal component of the menhaden, *Brevoortia patronus*, is very sensitive to pH as evident by the rapid oxygen desaturation below pH 7.0. The major component of the stripped bass, *Morone saxatilis*, also exhibits a strong Root effect. In contrast, the gafftopsail catfish, *Bagre marinus*, lacks a Root effect. Even at pH 5.4, this hemoglobin remains 86% oxygen saturated. The lack of a Root effect is usually associated with the absence of a swimbladder in the fish.

**DISCUSSION**

Fish are unique in that most species exhibit hemoglobin polymorphism which appears advantageous for adaptive or survival purposes within varying habitats. Previous studies from our laboratory focusing upon several species of North Pacific salmon have clearly indicated the functional differences of these polymorphic hemoglobin components (Harrington, 1985, 1986; Sauer and Harrington, 1988).

The fish species obtained from the waters of the Gulf of Mexico represent a diverse cross-section of fish. All the samples collected in this study came from relatively shallow waters (6 m) up to 100 m. Most species were found distributed over this range. Several exceptions included the short big eye, *Pristigenys alta*, and the pearly razorfish, *Hemipteronotus novacula*, which were obtained in waters 100 m deep.

Of the 13 species collected, three species, the gafftopsail catfish, *Bagre marinus*, the hardhead catfish, *Arius felis*, and spot, *Leiotesmus xanthurus*, have only one hemoglobin component as determined by starch-gel electrophoresis at pH 8.6. Several other species possess one major hemoglobin component and minor components. These are the pearly razorfish, *Hemipteronotus novacula*, the dwarf sand perch, *Diplectrum bivittatum*, and the striped bass, *Morone saxatilis*. Three other species, the jack cravelle, *Caranx hippos*, the Atlantic bumper, *Chloroscombrus chrysurus*, and the short big eye, *Pristigenys alta*, possess two major components that are electrophoretically distinct (Fig. 1). Initial mol. wt studies using Sephadex G-100 gel filtration chromatography indicated that all these distinct hemoglobin components from these fish species have mol. wts similar to human hemoglobin A. Co-elution with hemoglobin A (Fig. 2) resulting in a single symmetrical elution profile, also strongly
suggests little or no dimerization occurs with these fish hemoglobins. This also indicates that rehybridization of the different globin chains is not likely to occur intracellularly.

The isothermal denaturation of each of these fish hemoglobins in the presence of urea clearly indicates significant differences in their conformational stabilities and resistance to polypeptide unfolding. Spectral analysis of the unfolding of these fish hemoglobins was carried out in the Soret region (450–350 nm). This region is sensitive to changes in the heme moiety environment buried within the globin chains of these proteins. Figures 3A and 3B which exhibit large decreases in the absorbance spectra at 413 nm and the resultant blue shifts of wavelength maxima to 395–396 nm provide evidence of the extensive degree of hemoglobin unfolding that occurs with increased concentrations of urea. The observed maximum shift to 395 nm is associated with the release of the heme moiety from the native protein. Notice should also be taken of the absorbance spectra wherein no clearly defined isosbestic points are to be found. This suggests that the unfolded states of these hemoglobins are not the same, or that the known aggregation of the heme moiety upon unfolding and release may complicate the interpretation of the spectra (Elbaum et al., 1974).

The urea concentration at which 50% of these hemoglobin molecules are unfolded (D1/2) differs considerably among the species in the study (Table 1). These unfolding midpoints range from the low resistant hemoglobins of the ground mullet (D1/1 = 2.7 M and 2.8 M) to the more resistant hardhead catfish (D1/2 = 5.8 M) and gafftopsail catfish (D1/2 = 6.5 M). It should be noted that the transition profiles (Figs 4A and 4B) associated with the native to unfolded protein equilibria are different in the steepness of their transitions. Empirically, the sharper transitions are usually related to an increase in cooperativity within the unfolding process, assuming a single step transition (Native ↔ Denatured).

No clear pattern of denaturation behavior is apparent from these hemoglobins examined. Likewise, it is unfortunate that little data are available on the extent of subunit dissociation occurring prior to the secondary structural changes. Subunit dissociation studies of several of these fish hemoglobins are presently underway in our laboratory.

In addition to the usual role of transporting oxygen to tissues, hemoglobin often carries out other important functions. One of these functions is to provide the swim bladder with oxygen against a pressure gradient. This function assists the fish in controlling its buoyancy. In many species of fish the presence of different kinds of hemoglobin components permits sufficient oxygen transport by one hemoglobin component (usually the more cathodal component) even under acidic conditions while also utilizing a Root effect hemoglobin (anodal component) within the rete mirabile of the eye and/or the swim bladder (Johansen and Weber, 1976; Farmer et al., 1979). Some fish, like the spot fish, possess a single hemoglobin component (Bonaventura et al., 1976), or a major hemoglobin component, like the striped bass within this study, also exhibit a strong Root effect. Under these conditions, the equilibrium is shifted towards the more stable T conformational state of these tetramers (Bunn and Forget, 1986).

REFERENCES


