Studies on Myoglobins of Salmonids*¹

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The existence of and the relative mobilities of myoglobin (Mb) in both the heart muscles and the lateral line red muscles of rainbow and cutthroat trout and in the heart muscles of 5 species of Pacific salmon have been demonstrated by starch gel electrophoresis. Of the salmon, the pink alone has significant amounts of Mb in the lateral line red muscle. All salmonid species examined here exhibited only a single Mb component except for the coho which revealed two components.

Mb was isolated from the heart muscles of sockeye, coho, and chinook in yields of 62, 27, and 21 mg respectively per 100 g wet weight of tissue. The absorption spectra of 5 derivatives prepared from the Mb's isolated from the 3 species of salmon were hardly distinguishable from those of other piscine Mb's. The oxygen affinity curves of Mb's from the 3 species of salmon were affected only slightly by pH in the range of 6-8. At pH 7.0 and 15°C, the oxygen pressure at half saturation was measured to be 0.8, 1.1, and 1.8 mmHg for Mb's from coho, chinook, and sockeye respectively. The oxygen affinity of chinook Mb was to some extent increased by copper sulfate and slightly decreased by both 2,3-diphosphoglyceric acid and ATP.

Many of the studies on fish myoglobin (Mb) have been carried out on scombroid fishes. Large quantities of Mb in teleost fishes are found in the red muscles, reaching as high as 1-2% on a wet weight basis in the deep-seated red muscles of some scombroid fishes.¹⁾ The same investigators described a number of properties of tuna Mb, including spectral properties, free boundary electrophoretic characteristics and oxygen dissociation curves.²⁻⁴⁾ Amino acid compositions of the Mb's of *Thunnus orientalis*,⁵⁾ of *Thunnus albacares*,⁶⁾ of *Katsuwonus pelamis* and *Parathunnus sibi*⁷⁾ and the N-termini of Mb's from *Thunnus orientalis* and *Katsuwonus vagans*⁸⁾ have been reported. The isolation and some chemical properties of Mb from the heart muscle of chinook salmon was reported.⁹⁾

The present communication describes some properties of Mb's from 2 species of *Salmo* and 5 species of *Oncorhynchus*.

Materials and Methods

Materials Heart, lateral line muscle, kidney, spleen and liver tissues were collected from the following 5 species of Pacific salmon and two species of trout immediately upon death and stored deep frozen: the sockeye salmon *Oncorhynchus nerka*, the chum salmon *O. keta*, the coho salmon *O. kisutch*, the chinook salmon *O. tshawytscha*, the pink salmon

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O. gorbuscha, the rainbow trout *Salmo gairdneri*, and the cutthroat trout *S. clarki*. The hearts were sliced open and the excess blood washed off with cold distilled water. Whole blood was obtained from the caudal artery, the erythrocytes separated by centrifugation and washed with 1% sodium chloride solution.

Preparation of protein samples for starch gel electrophoresis Hemoglobins were prepared by mixing the packed erythrocytes with 1–2 volumes of a slurry of Sephadex G-25 according to a method used previously¹⁰⁾ and adapted from a technique first described by KOCH *et al.*¹¹⁾

Tissues were homogenized with equal volumes of cold, 0.05 ionic strength phosphate buffer of pH 7.5, centrifuged at $40,000 \times g$ for 10 min, and the supernatant used directly for electrophoresis. Starch gel was prepared in 0.023 M borate buffer, pH 8.5, and electrophoresis was carried out using 0.3 M borate buffer, pH 8.5, as the bridge buffer solution.¹⁰⁾ Both the gel and bridge buffers contained 0.01% potassium cyanide and potassium ferricyanide.¹²⁾ Electrophoresis was performed in the refrigerator for 2.5 hr at a potential gradient of 13 v/cm. The heme proteins were visualized with *o*-dianisidine¹³⁾ and the total proteins with amido black dye either on a previously unstained gel or those stained with *o*-dianisidine.

Isolation of Mb Mb was isolated in its met-form from the heart muscle of sockeye, coho, and chinook salmon by a slight modification of the method of MATSUURA *et al.*²⁾ All procedures were carried out at around 4°C. About 50 g of heart muscle was minced and homogenized with 50 ml of cold distilled water. After an hour, the homogenate was centrifuged for 15 min at $40,000 \times g$. The procedure was repeated immediately with 25 ml of water and the two extracts were combined. A saturated solution of monobasic lead acetate was then added to the extract until white precipitates no longer formed. Following centrifugation for 15 min at $40,000 \times g$, a saturated solution of sodium pyrophosphate was added to the supernatant till precipitation ceased and the mixture was re-centrifuged. The fraction precipitating between 55 and 85% saturated ammonium sulfate was collected, dissolved in a small amount of water, and dialyzed against several changes of cold distilled water for 2 days. After the insoluble material was removed by centrifugation, the supernatant was passed through a 1.5×115 cm column of Sephadex G-50 equilibrated with 0.1 M phosphate buffer, pH 7.0. Five ml fractions were collected at a rate of 0.5 ml per min and each fraction was checked for Mb by starch gel electrophoresis. The Mb rich fractions were combined, precipitated with ammonium sulfate at 85% saturation, dissolved in a little water, dialyzed for one day against several changes of distilled water, and finally subjected to starch block electrophoresis in 0.1 M phosphate buffer, pH 8.0, for 18 hr at a potential gradient of 2 v/cm. The Mb zone was cut out from the starch block tray, extracted with cold distilled water and analyzed for properties described in this communication after homogeneity was confirmed by starch gel electrophoresis.

Spectrophotometry Absorption spectra of Mb's from the 3 species of salmon were measured with a Beckman DU spectrophotometer in a 0.1 M phosphate buffer of pH 7.0 according to the method of MATSUURA *et al.*²⁾ Mb derivatives were prepared by the method of BOWEN.¹⁴⁾ Millimolar extinction coefficients of the derivatives at $\lambda_{\max}$ and $\lambda_{\min}$ were calculated from the ϵ value of 11.3 for cyanmetmyoglobin (MMbCN) at 540 nm.¹⁵⁾

Oxygen dissociation curve The metmyoglobin (MMb) isolated from the tissues was converted to its reduced form in the cold with trace amounts of sodium hydrosulfite and then to the oxy-form by gentle shaking in contact with air.⁴⁾ The amount of sodium hydrosulfite used was carefully controlled so that it was 0.04% or less according to SNYDER *et al.*¹⁶⁾ and MATSUURA *et al.*¹⁷⁾ Oxygen affinity was measured by a slight modification of a tonometric method used by HASHIMOTO *et al.*¹⁸⁾ Four ml of Mb solution was pipetted into a 317 ml tonometer attached to a 2 mm quartz cell. The tonometer was evacuated and flushed with nitrogen. This procedure was repeated several times and the tonometer finally evacuated. A calculated volume of air was injected into the tonometer and the entire assembly was rotated horizontally at 30–35 rpm in a constant temperature water bath held at 15°C. The degree to which Mb was saturated with oxygen was measured by the change in absorption at 555 nm. The formation of MMb in the Mb solution during the oxygen equilibrium was negligible as examined spectrophotometrically. The dissociation curves were independent of Mb concentration within the 0.12–0.28% range used. A 0.1 M phosphate buffer of pH 7.1 was used except where indicated otherwise.

Effect on oxygen dissociation

Copper To measure the effect of Cu^{2+} on oxygen dissociation, a 0.1 M solution of Tris (hydroxymethyl) aminomethane (Tris) at pH 7.1 was used in place of phosphate buffer. No observable difference in the dissociation curve was introduced by Tris buffer. To 3.9 ml solutions of oxymyoglobin (MbO_2), 0.1 ml solutions of copper sulfate of various concentrations were added and the oxygen dissociation curve was measured against a control. The sulfate anion was also tested using sodium sulfate.

2, 3-Diphosphoglyceric acid 2, 3-Diphosphoglyceric acid (DPG), obtained as the pentacyclohexyl ammonium salt tetrahydrate, was converted to the free acid by a slight modification of the method described by FRANKLIN BUNN *et al.*¹⁹⁾ To 20 g of 100–200 mesh Dowex-50 was added 225 mg of DPG dissolved in 12.5 ml water, the mixture shaken for 30 sec, allowed to settle and brought to 100 ml with water. The supernatant was filtered through a fine sintered glass funnel, lyophilized and brought to 10 ml with water. The concentration of DPG was determined by titration with sodium hydroxide. To 3.9 ml solutions of MbO_2 were added 0.1 ml solutions of DPG of the appropriate concentrations to achieve the desired molar ratios with Mb.

ATP To 3.9 ml solutions of MbO_2 were added 0.1 ml solutions of ATP of the appropriate concentrations and the oxygen dissociation was measured as above.

Results and Discussion

Starch gel electrophoretic patterns of heme proteins In all salmonid species shown in Fig. 1, Mb components appear as fast moving anodal zones and are distinctly separated from their respective multiple hemoglobin (Hb) zones. In the lateral line red muscle and the heart muscle of cutthroat trout (Fig. 1A), Mb's of similar mobilities are found in about the same concentration. Similar Mb profiles are found in rainbow trout (Fig. 1B). The mobilities of the Mb's from the two species of *Salmo* are also the same. The relative mobilities of the Mb's of the two *Salmo* species and the five *Oncorhynchus* species can be gleaned from Fig. 1C, which shows that the Mb's of chum salmon migrate at the same rate as those from the *Salmo*, and from Fig. 1E, which shows the comparative mobilities of the Mb's of Pacific salmon.

The virtual absence of Mb in the lateral line red muscles of most Pacific salmon species and in the spleen and kidney tissues of all five species is shown in Fig. 1D, using sockeye salmon as an example. In view of the finding of large amounts of Mb in the lateral line red muscles of the closely related *Salmo* species, the absence of this heme protein from the same tissues of sockeye, chum, coho, and chinook salmon in quantities not detectable electrophoretically was indeed surprising. Pink salmon alone has significant amounts of Mb in the lateral line red muscles. This study not only confirms the absence of Mb in the red muscle of chum salmon as reported by HASHIMOTO *et al.*¹⁸⁾ but also shows that this

Fig. 1. Starch gel electropherograms of salmonid Mb's. The anode is to the right. All zones to the right of Hb represent Mb's.

characteristic is more widespread in Pacific salmon.

Wide mobility differences exist in the Mb's from the heart tissues of the 5 species of salmon unlike what may be expected within closely related species (Fig. 1 E). Under the conditions of electrophoresis only a single component is visible in the pink, sockeye, chum, and chinook salmon and in the rainbow and cutthroat trout (Figs. 1 B and E). Coho salmon alone has 2 molecular species of Mb in the heart tissue (Fig. 1 E). More than one molecular species of Mb has been reported in bigeye tuna, *Parathunnus sibi*³⁾ and in tuna, *Thunnus orientalis*.²⁰⁾

Yield of Mb From 100 g of heart muscle, 62, 27, and 21 mg of Mb were isolated respectively from the sockeye, coho, and chinook salmon. The Mb content of salmon is very low compared to the 1–2% found in the red muscle of tunny fish.²⁾

Absorption spectra The absorption data in the visible region of Mb's from salmon are shown in Table 1. Except where noted, all derivatives of Mb prepared from the 3

Table 1. Millimolar extinction coefficient, ϵ , of the derivatives of three species of salmon Mb's, as calculated on the basis of Mb concentration, which was determined by using the ϵ of MMbCN at 540 nm = 11.3 by DRABKIN¹⁸⁾

Deriv.		Sockeye		Coho		Chinook		Horse ¹⁴⁾	
		λ	ϵ	λ	ϵ	λ	ϵ	λ	ϵ
		nm		nm		nm		nm	
MbO ₂	min (m')	505	6.9	510	7.1	506	7.0	510	5.5
	max (β)	542	11.5	540	12.0	541	13.7	544	14.6
	min (m)	561	7.1	561	8.8	561	8.6	564	8.5
	max (α)	576	10.2	576	11.4	575	11.6	582	15.1
	ratio β /m	1.62		1.36		1.59		1.72	
	ratio α /m	1.44		1.30		1.35		1.78	
MbCO	min (m')	492–3	5.7	496	7.0	497	6.5	500	5.29
	max (β)	539	14.3	537	12.7	538	15.5	540	14.85
	min (m)	555	11.4	555	11.4	555	12.4	560	10.57
	max (α)	567	13.4	567	12.3	567	14.4	577	12.95
	ratio β /m	1.25		1.11		1.25		1.40	
	ratio α /m	1.18		1.08		1.16		1.23	
DeoxyMb	min (m)	480	4.3	483	5.3	480	4.7	480	3.92
	max (α)	555	13.1	556	12.4	555	13.7	555	12.92
	ratio α /m	3.05		2.34		2.91		3.30	
MMb	max (β)	501	9.6	500	10.2	500	9.6	500	9.8
	min (m)	604–5	3.2	604	3.7	600	3.0	590	3.0
	max (α)	631	4.2	630	4.9	631	4.1	630	3.7
	ratio β /m	3.0		2.76		3.20		3.27	
	ratio α /m	1.31		1.32		1.37		1.23	
MMbCN	min (m)	504–5	7.6	504	8.5	504	7.4	510	6.87
	max (α)	540	(11.3)	540	(11.3)	540	(11.3)	540	(11.3)
	ratio α /m	1.49		1.33		1.53		1.64	

species of salmon have almost the same absorption maxima and minima characteristic of that particular molecular species. Compared to the two other species of salmon, significant shifts are found in the $\lambda_{\min}$ (m') of coho MbO₂, the $\lambda_{\min}$ (m') of sockeye carbonylmyoglobin (MbCO), and the $\lambda_{\min}$ (m) of chinook MMb. The ratio of maxima to minima (α/m , β/m) for each derivative of Mb from coho was slightly lower than those from sockeye and chinook salmon, except that the ratio of α/m of MMb was slightly higher than that from sockeye salmon.

Compared to the chinook salmon values of BROWN *et al.*⁹⁾, this study shows that the $\lambda_{\max}$ of MbCO, deoxymyoglobin (Deoxy Mb) and MMb from the same species are shifted very slightly (1–2 nm) towards the shorter wavelengths. Despite its taxonomic distance, the absorption spectrum of Mb from bigeye tuna is very similar to that from salmon. It is known that in fish and mammals the corresponding absorption spectra of MMb and Deoxy Mb in the visible region are similar, and that those of MbO₂ and MbCO are quite different.²⁾ Compared to the corresponding derivatives from horse, the $\alpha_{\max}$ and $\beta_{\max}$ of MbO₂ from salmon are not only shifted several nm to the shorter wavelengths, but also the molar extinction coefficients at the two maxima are considerably lower. Likewise, $\alpha_{\max}$ of the salmon MbCO is shifted 10 nm towards the shorter wavelengths. In MbO₂ and MbCO from salmon, the $\lambda_{\max}$ are almost identical to the corresponding derivatives of Hb from fish and mammalian sources.

In the near ultraviolet region (Table 2), no species specific significant differences exist in the $\lambda_{\max}$ of MbO₂, MbCO, Deoxy Mb, MMb, and MMbCN from the coho, sockeye, and chinook salmon. A slight difference in $\lambda_{\max}$ of 1–2 nm was noted for chinook salmon between this study and that of BROWN *et al.*⁹⁾ Considerable differences were noted between species in the millimolar extinction coefficient at the SORET band. In all Mb derivatives examined, those from coho displayed by far the highest extinction values of the 3 species. The MbCO and MMb from chinook salmon were notable for their exceptionally low values compared to sockeye and coho salmon.

As indicated above, the absorption spectra of Mb derivatives prepared from heart muscles of *Oncorhynchus* have a number of characteristics similar to those from other fishes.^{2,7,9)}

Table 2. Millimolar extinction coefficients, ϵ , of the SORET bands of salmon Mb's

Deriv.	Sockeye		Coho		Chinook	
	λ	ϵ	λ	ϵ	λ	ϵ
	nm		nm		nm	
MbO ₂	414	95	413	147	412	109
MbCO	420	224	420	240	419	151
DeoxyMb	430	119	431	147	430	104
MMb	406	191	407	213	406	159
MMbCN	420	105	420	134	419	120

Oxygen dissociation curve The oxygen affinity of Mb from chinook salmon was independent of concentration as was reported for tuna Mb.⁴⁾ The oxygen dissociation curves of Mb's from sockeye (Fig. 2), coho (Fig. 3), and chinook salmon (Fig. 4) are affected only slightly by pH in the range from about 6 to 8. In both sockeye salmon and coho salmon, the oxygen affinity tends to decrease with higher pH while the opposite effect is produced in the Mb from chinook salmon. In this respect, the Mb from chinook salmon resembles that from tuna.⁴⁾ Of the three salmon Mb's, that from sockeye salmon shows the largest change in affinity with pH, the oxygen pressure at half saturation (P_{50}) changing from 1.5 mmHg at pH 6.24 to 2.1 at pH 7.92. The value of $-\Delta \log P_{50}/\Delta \text{pH}$ for sockeye Mb was -0.1 , indicating a reversed BOHR effect. Values of 0.03 for human Mb²¹⁾ and 0.09 for horse Mb²²⁾ have been reported.

Fig. 2. Effect of pH on the oxygen dissociation curve of sockeye Mb (0.1 M phosphate buffer, 15°C).

Fig. 3. Effect of pH on the oxygen dissociation curve of coho Mb (0.1 M phosphate buffer, 15°C).

Fig. 4. Effect of pH on the oxygen dissociation curve of chinook Mb (0.1 M phosphate buffer, 15°C).

The P_{50} value at 15°C and at about pH 7.0 for coho, chinook, and sockeye salmon Mb are 0.8, 1.1, and 1.8 mmHg respectively. The latter two values are slightly higher than those of many other species.²³⁾ The P_{50} value of coho Mb compares with 0.6 mmHg for *Thunnus orientalis*⁴⁾ and 0.9 mmHg for *Thunnus thynnus*.²⁴⁾ The n values of HILL's equation calculated from Figs. 2, 3, and 4 were 1.0 for the Mb's of sockeye and chinook salmon and 1.2 for that of coho salmon.

Effect of copper on oxygen dissociation curve Slight decreases in affinity of chinook Mb for oxygen was noted with sodium sulfate at concentrations of 1, 10, 20, and 100 ppm (Fig. 5). At these concentrations the P_{50} values were increased by about 0.1–0.4 mmHg compared to the control. Tuna Mb is only very slightly affected by salt.⁴⁾ At all concentrations tested, copper sulfate increased the affinity of chinook Mb for oxygen (Fig. 6). Compared to the control, the P_{50} value was decreased by 0.05, 0.1, and 0.35 mm of mercury at copper sulfate concentrations of 1, 10, and 20 ppm respectively. If not for the opposing effect of the sulfate ion, these increases in affinity might have been more pronounced. At a concentration of 20 ppm, however, the shape of the equilibrium curve had been changed. At 100 ppm, the Mb was completely precipitated. The n value near P_{50} was found to be 1.0 at copper sulfate concentrations of 1 and 10 ppm decreasing to 0.9 at 20 ppm.

The toxicity of copper to fish is well known and has been reviewed by DOUDOROFF *et al.*²⁵⁾ and by MCKEE *et al.*²⁶⁾ At least *in vitro*, copper ion increases the affinity of chinook Mb for oxygen much like its effect of salmon Hb (TSUYUKI, unpublished). While the

Fig. 5. Effect of sodium sulfate on oxygen affinity of chinook Mb (0.1 M Tris-HCl buffer, pH 7.10, 15°C).

Fig. 6. Effect of copper sulfate on the oxygen affinity of chinook Mb (0.1 M Tris-HCl buffer, pH 7.10, 15°C).

concentration levels of copper at which increased affinity is first noted for Mb is approximately 500 fold higher than the chronic toxicity level to *Daphnia magna*,²⁷⁾ the effective level *in vivo* is not known. An increase in affinity suggests that copper may affect the respiratory function of Mb by interfering with the unloading of oxygen.

Effect of organo-phosphates on oxygen dissociation curve The oxygen affinity of chinook Mb was lowered progressively as the molar ratio of DPG to Mb was increased from 0.1 to 1.8 (Fig. 7). At the highest ratio, the P_{50} value was increased by about 0.5 mmHg or 45% over the control. When the ratio was increased to 3.5, all the Mb was precipitated. The n value of HILL's equation was 0.9–1.1, a figure similar to that of the control. It is interesting to note that at a molar ratio of DPG to Mb comparable to that which resulted in a 45% increase in P_{50} value, a figure of roughly 400% increase was observed for cichlid Hb at pH 7.2.²⁸⁾ The same study showed that cichlid red cells contained 15 times more ATP than DPG, compared to human red blood cells which contained 5 times more DPG than ATP.²⁹⁾

At a molar ratio of ATP: Mb from 0.6 to 3.8, the affinity of Mb for oxygen was decreased progressively in much the same manner and extent as with DPG, reaching at the highest ratio a P_{50} value 55% higher than the control. Unlike DPG, however, no precipitation of Mb was observed even at the highest ratio tested. The n value of HILL's equation was 1.0–1.2. For cichlid Hb, at a comparable molar ratio of ATP: Mb, the P_{50} value was increased approximately to 9 fold over the control.²⁸⁾ The relative difference between

Fig. 7. Effect of DPG on the oxygen affinity of chinook Mb (0.1 M Tris-HCl buffer, pH 7.10, 15°C).

Fig. 8. Effect of ATP on the oxygen affinity of chinook Mb (0.1 M Tris-HCl buffer, pH 7.10, 15°C).

DPG and ATP in reducing the oxygen affinity in that study was not apparent in chinook Mb.

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