Overproduction of α Chains Provides a Proton-insensitive Component to the Bluefish Hemoglobin System*

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Expression of α and β chains and their post-translational assembly into αβ₂ tetramers is fundamental to the formation and function of most vertebrate hemoglobins. There is a strong evolutionary bias that favors expression of equal amounts of the two types of chains, because cooperativity, pH sensitivity, and anionic control of function occurs only for the αβ₂ tetramers. Remarkably, an over-production of α chains, as in the pathological condition known as β thalassemia in humans, is adaptive rather than pathological in the bluefish hemoglobin system. The thalassemia of the bluefish is a novel means of providing for oxygen uptake and delivery when low pH conditions incapacitate the highly pH-sensitive Root effect hemoglobins of the fish. Although fish often have pH-insensitive along with highly pH-sensitive hemoglobins, having pH-insensitive α chain monomers in circulation is an unusual structural variation. The role of bluefish α chains in oxygen transport is enabled by their remarkably lower oxygen affinity relative to human α chains. This is the first reported case of a thalassemic condition that is maintained in a species as an adaptive advantage.

The bluefish (Pomatomus saltatrix) is a highly active predatory fish that is distributed worldwide. Movement of large schools of bluefish appears to be regulated by water temperature, with movements northward and inshore in the spring and offshore in the late summer months (1). Bluefish are considered to be among the most ferocious and blood-thirsty fish in the sea, leaving in their wake a trail of dead and mangled menhaden, herring, mackerel, and other fishes on which they prey. Rapidly swimming bluefish have high respiratory oxygen demands and can experience blood pH changes during periods of high activity. They are powerful swimmers. They have been reported to attain velocities up to 3.8 m s⁻¹ when startled and to travel at average speeds of 0.2–1.6 m s⁻¹. Even small bluefish can sustain swimming speeds of 4.0–4.6 body lengths/s for extended time periods (2).

The bluefish Hb system, like that of the trout (3) and eel (4), contains some tetrameric Hbs in which oxygen binding is highly pH-sensitive (Root effect Hbs) and other Hbs with little or no pH sensitivity. The bluefish uses the highly pH-sensitive Root effect Hbs, not only for oxygen transport to respiring tissues, but also for proton-driven oxygen unloadings into the swim bladders to create neutral buoyancy at varied depths. Bluefish have been reported to load and unload oxygen into and out of their swim bladders at the fastest rates reported for any fish (5). Fish often have a number of electrophoretically and functionally distinct Hbs in which varied oxygen affinities are potentially helpful in oxygen uptake and utilization amid changing environments. The expression of multiple types of α and β chains and their assembly into functionally distinct αβ₂ tetramers makes these functional adaptations possible. The isolated chains do not show the elegant allosteric properties of the assembled tetramers (6).

As reported by Root (7) and Scholander and Van Dam (8) and discussed in relation to molecular adaptations of respiratory proteins (9–11), some fish, including the bluefish, have extremely pH-dependent Hbs that carry out functions in addition to normal oxygen transport. The Root effect Hbs of fish are so greatly affected by protonation that they serve as proton-driven pumps that deliver oxygen to specialized tissues even against large pressure gradients. In vitro, air-saturated Root effect Hbs can release as much as 80% of their bound oxygen as the pH is decreased from pH 8 to 6. When brought to low pH conditions in capillary networks, these fish Hbs can unload oxygen to the swim bladder and transport extra oxygen to the brain and eyes against large pressure gradients (8, 12–13). The structure/function relationships underlying this exaggerated Bohr effect have been the subject of extensive study (3, 14–18). As discussed elsewhere (15, 18), the Root effect derives from modification of the β-93 residue (normally Cys) and an ensemble of other structural modifications. The Root effect has not yet been reproduced in human Hbs modified by site-directed mutagenesis, despite intensive efforts to accomplish this feat (9).

Some fish erythrocytes contain, not only Root effect Hbs, but also Hbs that show little or no alteration in oxygen affinity associated with changes in pH. We and others believe it is an adaptive advantage for fish to express both pH-sensitive and -insensitive Hbs. This combination can provide a backup for oxygen uptake and delivery in cases where decreases in blood pH render the Root effect Hbs ineffective (3–4, 9, 14–21).

The pH-insensitive Hbs of fish carry a different net charge than the Root effect Hbs and typically migrate toward the cathode during electrophoresis. These cathodal fish Hbs, exhibiting little or no pH sensitivity, have also intrigued researchers and prompted intensive investigation. Residues that normally confer pH sensitivity have been altered in these Hbs. They typically lack the pH-sensitive histidine residue normally found at the β chain C terminus and have acetylated (pH-insensitive) α chain N termini. These modifications largely account for their pH insensitivity (3–4, 9).

As previously noted, the bluefish Hb system contains both pH-sensitive (Root effect) and pH-insensitive Hbs. As found in other fish, the bluefish Hbs that migrate toward the anode upon electrophoresis were found to be extremely sensitive to pH and to anionic cofactors, whereas the Hb of the bluefish that migrates toward the cathode is insensitive to pH and anions. Surprisingly, the cathodal bluefish Hb shows no cooperativity in oxygen binding (n = 1 in Hill plots). This unusual feature prompted us to undertake a more complete evaluation of the bluefish...
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Hb system. The results reported in this paper provide an explanation for the unusual lack of cooperativity of the cathodal bluefish Hb.

The pH-insensitive cathodal fish Hbs previously studied are cooperative α2β2 tetramers that are structurally distinct from the α2β2 tetramers of the pH-dependent anodal Hbs (3, 4). Our structure/function studies on bluefish Hbs showed that the bluefish system is unlike the previously studied fish Hb systems. In the bluefish, the pH-insensitive cathodal Hb is generated by an elevated production of α chains relative to β chains. The unpaired α chains lack the allosteric controls of the tetrameric ensemble and contribute to oxygen transport in a pH-insensitive manner. The overproduction of α chains is an unusual mechanism for generating an oxygen transport system containing both pH-sensitive and pH-insensitive Hb components.

The overproduction of α chains is pathological in humans, a condition known as β thalassemia. The human α chains have such high affinity that they do not deliver oxygen effectively to respiring tissues. As shown in the following results, the bluefish α chains have significantly lower oxygen affinity than human α chains. This functional difference enables the bluefish α chains to make a positive contribution to the oxygen transport process despite their non-cooperative nature. This is the first reported case of a thalasemic condition being conserved in a species because of the adaptive advantage it confers.

EXPERIMENTAL PROCEDURES

Bluefish, P. saltatrix (Linnaeus), were collected off the coast of Beaufort, NC. Blood samples were taken from juvenile fish estimated to be <1 year old, based on their lengths of 6–25 cm (1). The red blood cells were washed and then lysed by exposure to 0.01 M Tris buffer, pH 8.1, containing 0.005 M EDTA. The hemolysate was dialyzed overnight and stripped of cofactors by chromatography using a cation exchange column followed by a Sephadex G-25 column. The stripped hemolysate was concentrated to ~1 mM (heme) and stored under liquid nitrogen until used. Electrophoresis on cellulose acetate showed the presence of two Hb bands of equal intensity that migrated toward the anode (the anodal Hbs) and a smaller fraction that migrated toward the cathode (the cathodal Hb).

Separation of anodal and cathodal bluefish Hbs was accomplished using a fast phase liquid chromatography anion exchange system with Q-Sepharose Fast-Flow Resin (Pfizer Corporation). The effective separation of the anodal and cathodal Hbs was verified by electrophoresis on cellulose acetate and by electrospray ionization mass spectrometry (EIMS). After separation, the anodal and cathodal Hb components were subjected to functional and structural analysis. Cathodal trout Hbs (including trout 1 Hb) and anodal trout Hbs (including trout IV Hb) used in our comparative studies were similarly separated following methods previously described (3).

Oxygen binding experiments were done tonometrically according to the methods of Riggs and Wolbach (22). Oxygen binding affinities and cooperativity were determined from pH 6.0 to 8.3 with ~60 μM (in heme) Hb samples maintained at 20 °C in 0.05 M HEPES buffer, to which varied anionic cofactors were added to investigate allosteric effects.

Determination of the number of exposed sulfhydryl groups and their reactivity was done according to the method of Grassetti and Murray (23). Upon reaction with a sulfhydryl (SH) group, the sulfhydryl-specific reagent 4,4’-dithiopyridine generates the highly absorbing product 4-thiopyridine, which levels are quantified by spectral changes at 324 nm. In our studies, the 4,4’-dithiopyridine reagent was added at 2–4-

2 The abbreviations used are: EIMS, electrospray ionization mass spectrometry; SH, sulfhydryl.
second chromatographic peak (Fig. 1, Peak 2) had two closely eluting absorption peaks, indicative of heterogeneity. Electrophoresis confirmed that Peak 2 is not a single Hb component but is composed of the two anodally migrating Hbs. The trailing edge of Peak 2 was collected and concentrated and shown by electrophoresis and mass spectrometry to contain a single band corresponding to a single anodal Hb.

EIMS studies were carried out on the bluefish hemolysates and on separated chromatographic fractions (Fig. 1, Peaks 1 and 2) as described above. As shown in Fig. 2, Peak 1, containing the cathodal Hb, had a single α chain mass of 15,613 Da and, surprisingly, no β chain mass. The slower chromatographic band (Fig. 2, Peak 2) was found to contain approximately equal amounts of two types of α chains with masses of 15,613 and 15,675 Da, along with two types of β chains with masses of 16,366 and 16,403 Da. The existence of two α and β chain types was consistent with the presence of two types of anodal Hbs in the bluefish system. The trailing edge of Peak 2, which appeared upon electrophoresis to be a purified anodal Hb, had an α chain mass of 15,613 Da (identical to one of the α chains of Peak 2 and to that of the α chain of the cathodal Hb) and a β chain mass of 16,366 Da (data not shown). Finding a single α chain mass in Peak 1 without a partner β chain mass was very surprising, as was the mass equivalence of its single α chain to one of the α chains in Peak 2.

Fig. 2 also shows the EIMS pattern for the unfractionated bluefish hemolysate (containing both cathodal and anodal components). The ratio of α to β peaks in the unfractionated hemolysate was 100:31, much higher than for the Peak 2 fraction isolated from this hemolysate. The apparent excess of α chains in EIMS patterns of the unfractionated hemolysates of individual bluefish of varied sizes was one of several indications that the bluefish Hb system contains an excess of α chains.

Although EIMS measuring conditions can differentially amplify the mass peaks of specific types of proteins, the method can be used in a comparative manner if measuring conditions are kept constant, as was done in obtaining the results shown in Fig. 2.

EIMS patterns for hemolysates from the largest bluefish studied (30 cm in length) did not differ significantly from those examined from several smaller fish (9–15 cm). Bluefish 30 cm in length are typically 1.5–2 years old, but the 30-cm fish examined in this study may not be representative of the adult bluefish population. Size continually increases with bluefish age, with a maximum reported length of 115 cm. Further studies will be needed to determine if there is an age-dependent variation in Hb types expressed in the bluefish population.

Oxygen binding studies were carried out on cathodal bluefish Hb (Fig. 1, Peak 1) and on the mixed anodal bluefish Hbs (Peak 2). As shown in Fig. 3, the anodal and cathodal Hbs differed significantly in their pH sensitivity. Similar to other Root effect Hbs, air-equilibrated samples of anodal bluefish Hbs in the presence of organic phosphate cofactors uncharged oxygen as the pH was decreased to pH 7 and below. Only 38% of the oxygen binding sites of air-equilibrated anodal bluefish Hb were occupied under these physiologically relevant conditions, while the air-equilibrated cathodal Hb was 100% saturated from pH 8.5 to 6.5. Unfractionated hemolysates exhibited properties that reflected the contributions of both anodal and cathodal Hb components.

A few pH effect experiments similar to those of Fig. 3 were done with the small sample obtained by concentrating the trailing edge of Peak 2. As noted, electrophoresis and EIMS showed that this sample contained a single purified anodal Hb. Like the mixed anodal Hbs of Peak 2, the purified anodal Hb from the trailing edge of Peak 2 was saturated with oxygen at pH 8.5 and was only 40% saturated with oxygen when exam-
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FIGURE 4. Contrasting effects of pH and anions on oxygen affinity (log \( P_{50} \)) and cooperativity (\( n_{50} \)) of cathodal bluefish Hb (open symbols) and anodal bluefish Hbs (filled symbols). Oxygen equilibria were measured with Hbs at \( 60 \mu \)M heme in 0.05 M HEPES buffer at 20°C in the absence of cofactors (circles), with 0.2 \( M \) Cl\(^{-}\) (triangles), with 0.2 \( M \) PO\(_4\)\(^{3-}\) (inverted triangles), and in the presence of 600 \( \mu \)M inositol hexaphosphate (squares).

FIGURE 5. Representative Hill plots of oxygen binding by cathodal bluefish Hb at pH 6.8 (open circles) and at pH 8.3 (open squares) and by anodal bluefish Hbs (closed symbols) at pH 6.8 (circles), pH 8.3 (triangles), and at pH 6.8 in the presence of 600 \( \mu \)M inositol hexaphosphate (diamonds). Hbs were at \( 60 \mu \)M heme in 0.05 M HEPES buffer, 20°C.

Oxygen equilibria for the cathodal bluefish Hb and the mixed anodal bluefish Hbs were obtained using spectrophotometric methods (22). As shown in Fig. 4, the oxygen equilibria of cathodal bluefish Hb were unaffected by pH and anions. In contrast, oxygen binding by anodal bluefish Hb was strongly modulated by pH and anions. Inorganic anions, such as Cl\(^{-}\), had a relatively minor effect, while the more highly charged phosphate (PO\(_4\)\(^{3-}\)) and polyphosphate (inositol hexaphosphate) ions brought the anodal Hb to progressively lower oxygen affinity. The oxygen binding equilibria observed in the presence of organic phosphates most closely resembled the condition believed to exist in vivo.

Cooperativity (\( n_{50} \)) in oxygen binding by anodal bluefish Hb showed considerable pH dependence, most pronounced in the presence of the polyphosphate cofactor inositol hexaphosphate. The observation of \( n \) values <1 at low pH is typical of Root effect Hbs and is ascribed to pH-dependent chain heterogeneity in addition to pH-dependent stabilization of the low affinity conformation (3, 12, 18–20). In contrast, the Hill plots of oxygen binding to the cathodal bluefish Hb had \( n \) values of 1, showing the complete absence of cooperativity for this pH-insensitive Hb.

Compared with human Hb, the bluefish Hbs were found to be very prone to met-hemoglobin formation via autoxidation. High autoxida-
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bluefish is distinctive in having linear Hill plots with unity slopes. The data shown in Fig. 6 for the cooperative cathodal Hb of trout is representative of the cooperative oxygen binding of previously studied cathodal fish Hbs. Data from Fig. 6 and our other studies (not shown) of bluefish and trout Hbs are summarized in TABLE ONE to enable readers to make a direct comparison between the oxygen binding characteristics of the anodal and cathodal Hbs of bluefish and trout.

The preceding results showed that the cathodal bluefish Hb is not only non-cooperative but lacks both pH and anion sensitivity. This absence of allosteric behavior was determined to be due to the cathodal Hb being made solely of α chains, as indicated by the presence of a single α chain mass.

We considered that the cathodal Hb might be present in the circulation either as an α chain monomer or as an aggregate of α chains. We conclusively identified the bluefish cathodal Hb as an α chain monomer by analytical ultracentrifugation. Analytical ultracentrifugation results are presented in TABLE TWO. The sedimentation coefficients ($S_{20,w}$) and S/D estimates of molecular weight presented in TABLE TWO indicate that the anodal Hb fraction is composed of tetramers, whereas cathodal bluefish Hb contains only monomers.

SH group availability of the bluefish Hb components was studied, with results presented in TABLE TWO. These studies made use of the SH-specific reagent 4,4′-dithiopyridine, which generates highly absorbing 4-thiopyridine as a reaction product. The amount of 4-thiopyridine formed can be used to quantify the number of exposed SH groups (23). As found for human α chain monomers (6), the bluefish cathodal Hb was found to have two exposed SH groups/heme. This provides additional support for the conclusion that the cathodal Hb is composed solely of α chain monomers. Surprisingly, the anodal bluefish Hb was found to have four exposed SH groups, unlike human Hb tetramers that have only two exposed SH groups. This indicates the presence of a different steric configuration for SH groups in the tetramers of bluefish and human Hbs.

**DISCUSSION**

The foregoing results add another chapter to the intriguing story of adaptive structure/function relationships in fish Hbs. We have documented in this paper a remarkable respiratory adaptation in the bluefish Hb system that is based on an overproduction of α chains. Because the extra α chains do not have the allosteric responses characteristic of Hb tetramers (6), overproduction of α chains results in the presence of a non-allosteric, pH-insensitive Hb in bluefish blood. Production of excess α chains is a novel means of providing a pH-insensitive Hb to the bluefish circulatory system.

Fish Hbs have, for many years, attracted the attention of many researchers due to the wide spectrum of functional properties they exhibit. Adaptive Hb variations are apparently necessary, because fish are influenced to a great extent by their environments; e.g., variations in oxygen tension, temperature, and solute concentration greatly affect their physiology (3–4, 10–12). To accommodate these environmental shifts, their Hbs must function over a greater range of conditions than Hbs of higher vertebrates (4, 10, 25). Moreover, functions apart from the normal respiratory cycle are served by the Root effect Hbs of fish. These extremely pH-sensitive Hbs facilitate oxygen unloading to tissues such as the swim bladder, even at great depths, where the oxygen pressures in the swim bladder are very high (5–9). Gene duplications, common in fish, often give rise to the presence of many electrophoretically distinct Root effect Hbs in a single fish. The functional differences between these Hbs are often minor and have not been shown to have adaptive significance. The two anodal Root effect Hbs of the bluefish are a case in point. The production in some fish of pH-insensitive cathodal Hbs along with highly pH-sensitive Root effect Hbs is widely regarded as an elegant compensatory adaptation, one in which the pH-insensitive Hbs can provide a backup for oxygen uptake and delivery in cases where drops in blood pH render the Root effect Hbs ineffective.

The pH-insensitive fish Hbs previously studied are tetrameric proteins that have full cooperativity in oxygen binding (3–4). The pH-insensitive bluefish Hb is unusual in having no cooperativity in oxygen binding. Absence of cooperative interactions in the pH-insensitive cathodal Hb of the bluefish has been shown in this paper to be due to its monomeric nature. Its lack of cooperativity would make it less effective at oxygen unloading than other pH-insensitive cathodal Hbs. However,

**TABLE ONE**

| Hemoglobin component | $P_{50}$ and cooperativity ($n_{50}$) | ΔLog $P_{50}$ |
|----------------------|------------------------------------|---------------|
| Anodal bluefish      | pH 7.0                             | 1.10 (1.6)    |
| Anodal trout         | pH 8.5                             | 1.51 (1.4)    |
| Cathodal bluefish    | pH 7.0                             | 0.25 (1.0)    |
| Cathodal trout       | pH 8.5                             | 0.95 (2.2)    |

**TABLE TWO**

| Hemoglobin component | $S_{20,w}$ ($D_{20,w}$/P$_{50}$) | Exposed SH groups/heme |
|----------------------|----------------------------------|------------------------|
| Anodal bluefish Hb   | 4.05–4.15 (41,450)              | 0.8 ± 0.2              |
| Cathodal bluefish Hb | 1.65–1.75 (15,800)              | 1.9 ± 0.2              |

**FIGURE 6.** Representative Hill plots of oxygen binding by cathodal bluefish Hb (circles) and cathodal trout Hb (triangles) in the absence (open symbols) and presence (filled symbols) of 600 μM inositol hexaphosphate. Hbs were ~60 μM in heme in 0.05 M HEPES buffer, 20 °C.
its presence in the bluefish circulation serves the same function as in other fish containing pH-sensitive and -insensitive components. Its ability to bind and transport oxygen under low pH conditions would be preserved under conditions where the Root effect Hbs have extremely low oxygen affinity.

The bluefish α chains were found to have greater stability and lower oxygen affinity than human α chains, with \( P_{50} \) values at pH 7 of 1.8 (this paper) and 0.5 mm Hg (6), respectively. The nearly 4-fold higher \( P_{50} \) values for the bluefish α chains make them more able to unload oxygen and thus able to play a significant role in oxygen transport under physiological conditions.

It has been reported (6) that isolated human Hb chains have higher \( P_{50} \) values than human Hb tetramers, indicative of their lower affinity at high (90%) oxygen saturation. The fact that the tetramers of human Hb have a higher affinity in the R-state quaternary condition than that exhibited by the isolated chains is referred to as the quaternary enhancement effect. This effect has not previously been shown to occur in a fish Hb system, due largely to difficulties in generating and characterizing subunits of fish Hbs. The quaternary enhancement effect clearly occurs in the bluefish Hb system, as shown by the relative \( P_{50} \) values for α chain subunits and \( \alpha_2 \beta_2 \) tetramers of bluefish Hb (see Fig. 5).

There are two exposed SH groups on bluefish α chains, the same number as found on human α chains. Both α chain SH groups are buried in intersubunit contacts in the human Hb tetramer. The two exposed SH groups of oxygenated human Hb tetramers are contributed by β chains, because both α chain SH groups are buried (6). Oxygenated anodal bluefish Hbs, shown by ultra centrifugation to be tetrameric, have four exposed SH groups. We consider it probable that one of the α chain SH groups remains exposed in the tetrameric anodal Hbs of the bluefish, because other SH-bearing residues are exposed in the Root effect tetramers. We have shown elsewhere that these other SH groups could function in reactions with nitric oxide to form SNO-Hb (18).

The adaptive thalassemia exhibited by the bluefish Hb system is clearly a case where a simple elevation in levels of expression of α chains relative to β chains results in the production of structurally and functionally distinct Hbs that have complementary functions in vivo. It will be interesting to examine other biochemical systems for parallel cases of functional benefits arising from the co-existence of a protein monomer and a functionally distinct protein assembly containing the protein monomer.

In the human Hb system, overexpression of α chains relative to β chains is associated with pathological conditions and impaired oxygen transport. Pathology arises because the human α chains are unstable, lack cooperativity in oxygen binding, and do not unload oxygen effectively due to their high oxygen affinity. In contrast, the excess α chain production of the bluefish Hb system contributes a reasonably stable, although non-cooperative, protein monomer to the circulation. The lower oxygen affinity of the monomeric α chain of the bluefish relative to the human α chain allows it to play an important physiological role, because it can function in oxygen transport at low pH, where the Root effect Hbs of the fish are ineffective.

Having a non-cooperative α chain present as a back up system is a step removed from the more advantageous condition, found in many fish, where high pH sensitivity Hbs are paired with structurally distinct types of Hbs that are pH-insensitive but fully cooperative in their oxygen binding behavior. The pairing of pH-sensitive and -insensitive Hbs with fully expressed cooperative oxygen binding behavior facilitates effective oxygen transport over a very wide range of physiological and environmental conditions. The thalassemic condition maintained in bluefish as an adaptive advantage may represent an intermediate step in the evolutionary progression toward this more elegant solution.

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REFERENCES

1. Salerno, D. J., Burnett, J., and Ibara, R. M. (2001) J. Northw. Atl. Fish. Sci. 29, 31–39
2. Oliver, J. D., Avyle, M. J., and Bozeman, E. L., Jr. (1989) U.S. Fish and Wildlife Service Biological Report 82, 11-96
3. Brunori, M., (1975) in Current Topics in Cellular Regulation (Horecket, R. L., and Stadman, E. R., eds) Vol. 9, pp. 1–39, Academic Press, Oxford
4. Weber, R., Lykkeboe, E., and Johansen, K. (1976) J. Exp. Biol. 64, 75–88
5. Bentley, T. B., and Wiley, M. L., (1982) Environ. Biol. Fishes 7, 77–81
6. Antonini, E., and Brunori, M., (1971) Hemoglobin and Myoglobin in their Reactions with Ligands, pp. 195, 309–312, North-Holland Publishing Co., Amsterdam
7. Root, R. W. (1931) Biol. Bull. 61, 427–456
8. Scholander, P. F., and Van Dam, L. (1954) Biol. Bull. 107, 247–259
9. Nagai, K., Perutz, M. F., and Poyart, C. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 7252–7255
10. Weber, R. E. (1992) in Physiological Adaptations in Vertebrates (Wood, S. C., Weber, R. E., Hargens, A. R., and Millard, R. W., eds) pp. 257–277, Marcel Dekker, New York
11. Weber, R. E. (2000) in Hemoglobin Function in Vertebrates (Di Prisco, G., Giardina, B., and Weber, R. E., eds) pp. 23–37, Springer-Verlag Italia, Milano, Italy
12. Noble, R. W., Kwiatkowski, L. D., De Young, A., Davis, B. J., Haedrich, R. L., Tam, L.-T., and Riggs, A. F. (1986) Biochin. Biophys. Acta 870, 552–563
13. Pelster, B., and Weber, R. E. (1991) Adv. Comp. Environ. Physiol. 8, 51–77
14. Perutz, M. F. (1996) Nat. Struct. Biol. 3, 211–213
15. Mylvangangam, S., Bonaventura, C., Bonaventura, J., and Getzofl, E. (1996) Nat. Struct. Biol. 3, 275–283
16. Ito, N., Komiyama, N. H., and Fermi, G. (1995) J. Mol. Biol. 250, 648–658
17. Mazzarella, L., D’Avino, R., Di Prisco, G., Savino, C., Vitagliano, L., Moody, P. C., and Zagari, A. (1999) J. Mol. Biol. 287, 897–906
18. Bonaventura, C., Crumbhiss, A., and Weber, R. E. (2004) Acta Physiol. Scand. 182, 245–258
19. Giardina, B., Ascoli, F., and Brunori, M. (1975) Nature 256, 761–762
20. Bonaventura, C., Sullivan, B., and Bonaventura, J. (1976) J. Biol. Chem. 251, 1837–1876
21. Powers, D. A., Dalessio, P. M, Lee, E., and DiMichele, J. (1986) Am. Zool. 26, 235–248
22. Riggs, A., and Wohlback, R. J. (1956) J. Gen. Physiol. 39, 585–605
23. Grassetti, D. R., and Murray, J. F., Jr. (1967) Arch. Biochem. Biophys. 119, 41–49
24. Philo, J. S. (1997) Biophys. J. 72, 435–444