The Anodic Hemoglobin of Anguilla anguilla

MOLECULAR BASIS FOR ALLOSTERIC EFFECTS IN A ROOT-EFFECT HEMOGLOBIN*

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The functional and structural basis for the Root effect has been investigated in the anodic hemoglobin of the European eel, Anguilla anguilla. This hemoglobin exhibits a large Bohr effect, which is accounted for by oxygen-linked binding of seven to eight protons in the presence of GTP at pH 7.5. Oxygen equilibrium curves show nonlinear lower asymptote of Hill plots, indicating the occurrence of heme-heme interactions within the T state. Analysis of the curves according to the co-operon model (Brunori, M., Coletta, M., and Di Cera, E. (1986) Biophys. Chem. 23, 215–222) reveals that T state cooperativity is positive at high pH and in the stripped hemoglobin (where the T → R allosteric transition is operative) and negative at low pH and in the presence of organic phosphate (where the molecule is locked in the low affinity structure), indicating site heterogeneity. The complete amino acid sequence of eel anodic hemoglobin has been established and compared with that of other fish hemoglobins. The presence of the Root effect correlates with a specific configuration of the $\alpha_{b_2}$ switch interface, which at low pH would stabilize subunit ligation in the T state without changing the quaternary structure. We propose that the major groups involved in the binding of oxygen-linked protons in eel anodic hemoglobin are located on the $\beta$ chain and comprise His-HC3 at the C terminus, His-FG4 at the switch interface, and Lys-EF6 and the N terminus at the phosphate-binding site.

In contrast to mammals, fish show a large variation in the number of hemoglobin (Hb) components and in the mechanisms of oxygen binding modulation, which relates to their ability to adapt to widely different environmental conditions (1, 2). Conventionally, fish hemoglobins are divided into electrophoretically “cathodic” components (with high isoelectric points, pI $\geq 8.0$) and “anodic” ones (with low isoelectric points, pI $\leq 8.0$) that differ markedly in their functional properties (3). The European eel (Anguilla anguilla) may be considered as the simplest model with two functionally distinct major hemoglobins (4): a cathodic Hb with high oxygen affinity that is weakly affected by pH (with a small Bohr effect) (5) and an anodic Hb showing low oxygen affinity and large Bohr and Root effects similar to many anodic fish hemoglobins.

The Root effect of teleost fish is a large decrease in the oxygen affinity at low pH whereby the hemoglobin molecule cannot be fully saturated with oxygen even at very high oxygen tensions (6). The physiological role of Root-effect hemoglobins is to secrete oxygen into the swim bladder and the eye against high oxygen pressures following local acidification of the blood in a countercurrent capillary system (7). During respiratory or metabolic acidosis, oxygen binding to anodic hemoglobins may be hampered by their strong Bohr and Root effects, whereby oxygen transport may increasingly depend on the pH-independent and highly cooperative cathodic components that are commonly found in active fish species.

The Root effect originates from a strong, proton-dependent stabilization of the low affinity T (tense) quaternary structure relative to the high affinity R (relaxed) state (8). This inhibits the T → R allosteric transition and causes a drastic reduction in the Hill coefficient $n_5$ (the degree of cooperativity) to values close to unity or below. The T → R quaternary transition involves a rotation of the two $\alpha_{b_2}$ and $\alpha_{b_1}$ dimers relative to each other so that large conformational changes occur at the interdimer $\alpha_{b_2}\beta_2$ and $\alpha_{b_1}\beta_1$ interfaces, whereas the intradimer $\alpha_{b_1}$ and $\alpha_{b_2}$ interfaces remain virtually unaffected (9). Salt bridges and hydrogen bonds are broken in the transition from the T to the R state, resulting in the release of Bohr protons. These noncovalent interactions stabilize the T relative to the R state and act as constraints that determine the low affinity of the T state (10).

Accordingly, several mechanisms have been proposed, the molecular basis for the extraordinarily high stability of the T state in Root-effect hemoglobins is not yet fully understood. In the stereochemical model of Perutz and Brunori (11), the substitution of Cys-F9$\beta$ (in human HbA) with Ser (in Root-effect fish hemoglobins) was indicated as a crucial factor. However, site-directed mutagenesis experiments on human HbA showed that this substitution is not sufficient to induce the Root effect (12). More recent studies on the crystal structure of the carbonmonoxide form of spot Hb (Leistostomus xanthurus) have suggested that electrostatic repulsions between the positively charged residues (including the N terminus of the $\beta$ chain Lys-EF$\beta_6$, Arg-H21, and His-HC3$\beta$) protruding into the central cavity between the $\beta$ chains would destabilize the R state at low pH and induce the R → T transition that characterizes Root-effect hemoglobins (13). In spot Hb in the R state, the central cavity is narrower than in human HbA due to the presence of bulky Trp-NA3$\beta$ and Met-EPF$\beta_2$ residues (replacing Leu and Val in human HbA, respectively) and to a 3° larger rotation of the $\alpha_{b_1}$ relative to the $\alpha_{b_2}\beta_2$ dimer (a similar rotation is found also in the Hb1 of the antarctic teleost Pagochthia bernacchii (14)). However, this mechanism alone does not satisfactorily explain the absence of the Root effect in Hb1 of another antarctic nototheniid, Trematomus newnesi (15), which has all the necessary key residues except that Lys-EPF$\beta_6$ is replaced by Ala.

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The amino acid sequences reported in this paper have been deposited in the SWISS-PROT database under the accession numbers P80945 and P80946.

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(the same substitution is present in Nototenia angustata Hb1, which shows the Root effect (16)). A bulky Met residue at position E196 in T. neustes Hb1 has been suggested to interfere with the cluster formation (13), although a bulky Ile residue is present at this position in other Root-effect hemoglobins like those of goldfish or carp.

It appears that the search for the structural basis of the Root effect should not only concern the structural differences between the two end states, *i.e.*, the fully deoxygenated T and the fully ligated R state, but should also focus on the molecular mechanisms that allow the hemoglobin molecule to remain in the T state even when it is in the ligated form.

According to the two-state MWC model (17), cooperativity of oxygen binding arises from a concerted transition between the T and R states, both with noncooperative oxygen binding. The MWC model satisfactorily describes highly cooperative systems with a major allosteric equilibrium, which is the reason for its widespread utility in the analysis of ligand interactions. Nevertheless, T state crystals of human HbA bind oxygen cooperatively (18–20). The presence of complex positive (intradimer) and negative (interdimer) cooperative interactions has been revealed in the T state of human HbA (21, 22). Based on analysis of tetramer-dimer dissociation equilibria at different ligation stages, Ackers and co-workers (22) identified a third analysis of tetramer-dimer dissociation equilibria at different ligations for the study of the cooperative interactions occurring within the T state of tetrameric vertebrate hemoglobin.

We have analyzed the allosteric properties of the Root-effect anodic eel Hb following the co-operator model (25, 26) by assuming cooperative interactions within the T state and the quaternary structure (24) becomes apparent when the cooperativity originating from the major T \(\rightleftharpoons\) R allosteric equilibrium is inhibited as it is for Root-effect hemoglobins. As they may remain in the T state, even in the presence of oxygen, Root-effect hemoglobins represent ideal candidate systems for the study of the cooperative interactions occurring within the T state of tetrameric vertebrate hemoglobins.

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The abbreviations used are: MWC, Monod, Wyman, and Changeaux; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

**EXPERIMENTAL PROCEDURES**

**Purification of Anodic Hb—**Preparation of the hemolysate, separation from the cathodic Hb, and simultaneous stripping from organic phosphates by fast protein liquid anion-exchange chromatography were performed as described previously (5).

**Amino Acid Sequence Analysis—**Heme removal and precipitation of the globin chains were performed by the acid/acetone method (27). The α and β chains were separated by RP-HPLC on a Waters µBondapak C18 column (0.39 \(\times\) 30 cm) by a linear gradient of 70% acetonitrile (solvent B) in 50% acetonitrile, 0.3% trifluoroacetic acid (solvent A) as described (5). Reduction and carboxymethylation of SH groups were performed by incubating 2 mg of globin chain with 5 mM dithiothreitol in 6 M guanidine hydrochloride, 0.25 M Tris-HCl buffer, pH 9.0, for 50 min at room temperature followed by the addition of solid iodoacetic acid to a final concentration of 80 mM and adjustment of the pH with 1 M KOH. The reaction was stopped after 20 min by RP-HPLC. Alternatively, the carboxymethylation reaction was performed on the globin mixture containing both α and β chains and terminated by dialysis against double distilled water before RP-HPLC separation of the globin chains. The change in retention time on RP-HPLC of the globin chains after carboxymethylation indicated full alkylation at the SH groups. The alkylated chains were digested overnight by L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin at room temperature at an enzyme:protein weight ratio of 1:100 (w/w). Digestion of the α chain was performed in 2 μl urea.

Tryptic peptides were separated by RP-HPLC on a Waters µBondapak C18 column by a linear gradient of 70% acetonitrile, 0.08% trifluoroacetic acid (solvent B) in 2% acetonitrile, 0.1% trifluoroacetic acid (solvent A) as described (28). CNBr-generated fragments of the β chain were separated by RP-HPLC on a Nucleosil C18 column eluted by a linear gradient of 90% acetonitrile, 0.075% trifluoroacetic acid (solvent B) in 0.1% trifluoroacetic acid (solvent A). Selective cleavage at glutamic acid residues by Staphylococcus aureus protease V8 was performed in 0.1 M ammonium bicarbonate at 37 °C for 3 h (28). Decacylation at the N terminus of the chain was obtained by incubating the N-terminal blocked peptide with 30% trifluoroacetic acid at 55 °C for 3 h. Amino acid composition analyses were performed as described (29). The amino acid sequence was determined by automated Edman degradation using a pulsed liquid sequencer model 477A from Applied Biosystems equipped with a 120A analyzer for the detection of the phenylthiohydantoin-derivatives.

**Mass Spectrometry Measurements—**MALDI-TOF-MS was carried out on a Bruker Biflex instrument (Bruker-Franzen, Bremen, Germany) equipped with an ultraviolet laser at 337 nm. The samples dissolved in 0.1% trifluoroacetic acid were mixed with 2 μl of α-cyano-4-hydroxycinnamic acid, and 0.9 μl were applied to the target. 30–100 calibrated mass spectra were averaged.

**Oxygen Binding Studies—**Oxygen equilibria were measured in the pH range of 6.5–8.5 in 0.1 M HEPES buffer in the absence and presence of 0.1 M NaCl or 1 mM GSH at 20 °C and at a hemoglobin concentration of 200 μM heme. The pH values of the hemoglobin solutions were measured by a thermostated Radiometer BMS Mk2 capillary microelectrode. Cl− concentration was assayed by coulometric titration (Radiometer CMT 10).

In tonometrical oxygen equilibrium experiments (30), hemoglobin solutions were equilibrated to different oxygen tensions in thermostated tonometers (Eschweiler, Kiel, Germany) coupled to cased Wothhoff pumps for mixing humidified pure N2 (99.998%) with air or O2. To obtain the spectrum of the fully oxygenated hemoglobin at the end of the measurement, the pH of the sample (equilibrated with pure O2) was raised to pH 8.0–8.5 by the addition of solid HEPES salt. To minimize autoxidation during oxygen binding experiments, the methemoglobin-reducing system (31) was added to the hemoglobin solution (1 ml) as described by Imai (32) but using 1 μl of each reagent instead of 20 μl to minimize the possible effects of phosphates (as NADP or glucose 6-phosphate) on oxygen binding. No absorbance peak at 630 nm could be detected during the experiments, indicating that methemoglobin formation was negligible. To evaluate the Root effect, oxygen saturation was measured at different pH values in Eschweiler tonometers equilibrated with pure oxygen under the same experimental conditions used to measure the oxygen equilibria. The spectrum of the deoxygcnated hemoglobin was obtained after equilibration with pure N2. The value of 100% saturation was assigned to stripped hemoglobin at pH \(>8.2\).

Oxygen binding equilibria were also measured using a thin layer equilibration technique (modified gas diffusion chamber) utilizing cascaded Wothhoff pumps for mixing humidified pure N2 (99.998%) with
air or O₂ to obtain stepwise increases in oxygen tension (33, 34). The fractional saturation values were corrected for the incomplete oxygen saturation in the presence of 1 atm of oxygen due to the Root effect. The saturation achieved in the presence of oxygen (Y) was interpolated from the plot Y versus pH obtained in tonometrical experiments. Because of the rapidity of this method and the possibility to correct graphically for the small amount (<5%) of methemoglobin formed during the experiment (35), no reducing system was needed.

For detailed analysis of the allosteric interactions, diffusion chamber measurements at very low and high fractional saturation were carried out in the presence of the reducing system described above. Nonlinear least squares fitting was performed according to the co-operon model (25, 26). The binding polynomial for hemoglobin in the T state is as follows,

\[ P_T = (1 + 2K_o P_O_2 + iK_p P_O_2)^i \]  
(Eq. 1)

where \( K_o \) is the intrinsic ligand affinity and \( i \) describes the cooperative interactions within the T quaternary structure. Values of \( i \) above or below unity indicate positive and negative cooperativity, respectively. When \( i = 1 \), the T state binds oxygen with no cooperativity, and the model becomes identical to the classical two-state MWC model. The binding polynomial for a noncooperative R state with ligand affinity \( K_R \) is therefore that of the MWC model,

\[ P_R = (1 + K_R P_O_2)^i \]  
(Eq. 2)

According to these relations, the equation describing the fractional saturation in tetrameric hemoglobins (36) is,

\[ Y = \frac{L K_T P_O_2 (1 + 2 + iK_P P_O_2 + 3iK_P^2 P_O_2^2 + i^2K_P^3 P_O_2^3 + K_p P_O_2 (1 + K_p P_O_2)^i)}{L (1 + 2K_o P_O_2 + iK_p P_O_2)^i + (1 + K_R P_O_2)^i} \]  
(Eq. 3)

where \( L \) is the allosteric constant in the absence of ligand (17). The parameters of the equation were estimated from nonlinear least squares curve fitting. In addition, to minimize errors introduced by incomplete saturation or desaturation when equilibrating with pure oxygen or pure nitrogen, respectively, the absorbance values at zero (\( A_0 \)) and full saturation (\( A_{100} \)) were extrapolated from the data in the fitting procedure. In practice, the apparent saturation values (\( Y' \)) were calculated as follows,

\[ Y' = (A - A_0)/(A_{100} - A_0) \]  
(Eq. 4)

where \( A' \) and \( A_{100}' \) are the absorbance values measured in the presence of pure nitrogen and pure oxygen, respectively. The relationship between the true (\( Y \)) and the apparent (\( Y' \)) saturations is given by,

\[ Y = (Y' - Y_0)/(Y_{100} - Y_0) \]  
(Eq. 5)

or

\[ Y' = Y_0 + (Y_{100} - Y_0)Y \]  
(Eq. 6)

where \( Y_0 = (A_0 - A_0')(A_{100} - A_0') \) and \( Y_{100} = (A_{100} - A_0')(A_{100} - A_0') \) were the parameters fitted along with \( L, K_T, K_R, i, \) and \( Y \) is the expression of the fractional saturation according to Equation 3. Fitting was performed on Hill-transformed data, \( \log[Y/(Y - Y')] \) versus \( \log P_O_2 \) with equal weighting of data points. The curve fitter employed the method of Levenberg-Marquardt, and the standard errors on the parameters were estimated from the diagonal elements of the curvature matrix associated with the fit.

**RESULTS**

Structural Characterization—The anodic Hb of eel is a single component with an isoelectric point of 6.35, as indicated by isoelectrofocusing on polyacrylamide gel (5). Consistently, MALDI-TOF-MS measurements and RP-HPLC separation of the globin chains showed only two peaks corresponding to the \( \alpha \) and \( \beta \) chains that differ from those of the cathodic Hb (Fig. 1). Tryptic peptides of the S-carboxymethylated \( \alpha \) and \( \beta \) chains were purified by RP-HPLC as shown in Fig. 2. All peptides were sequenced and aligned by homology with the sequences of other fish globin chains. The complete amino acid sequence of the \( \alpha \) and \( \beta \) chains of the anodic Hb of eel is reported in Fig. 3 where the sequence portions elucidated by peptide sequencing are indicated. The intact \( \beta \) chain was sequenced up to Ile-20, whereas the \( \alpha \) chain was blocked and thus not accessible to Edman degradation. The sequence of the N-terminal peptide T1 of the \( \alpha \) chain was obtained after unblocking the N terminus as described under “Experimental Procedures.” The difference between the molecular mass of the \( \alpha \) chain measured by mass spectrometry (15,970 Da) and that deduced from the amino acid sequence (15,994 Da) is consistent with the presence of an acetyl group at the N terminus, as found in the \( \alpha \) chains of other fish hemoglobins. The molecular mass of the \( \beta \) chain measured by mass spectrometry (16,783 Da) is in agreement with that deduced from the sequence (16,764 Da) within experimental error. In the \( \alpha \) chain, trypsin failed to cleave at Lys-128, and the tryptic peptide from Phe-129 to Arg-140 was not recovered after RP-HPLC purification. The corresponding sequence was obtained after subfragmentation of the peptide T15 (extending from Ile-101 to Arg-140, sequenced up to Phe-130).
T15 was subjected to CNBr cleavage, followed by *S. aureus* V8 digestion. In this way, small fragments were generated (Ile-101–Met-107, Val-108–Met-112, and Thr-113–Glu-121) together with the larger V1 (Val-122–Arg-140). The peptide mixture was then subjected to automated Edman degradation, where the amino acid sequence of V1 could be unequivocally established by taking advantage of the early termination of the shorter peptides. Incomplete trypsin digestion was found at Lys-47, Lys-58, and Arg-93 in the *α* chain and at Arg-29 and Lys-59 in the *β* chain. No tryptic cleavage occurred after Arg-8 in the *β* chain probably because it follows three acid residues in the sequence. The fragments Thr-9–Lys-17 and Val-60–Lys-62 of the *β* chain were not recovered after RP-HPLC purification, and the corresponding sequences were obtained from N-terminal sequencing of the intact chain and from peptide T5, respectively. The *β* chain was also cleaved by CNBr, and the fragments were separated by RP-HPLC. The presence of two adjacent Arg residues (Arg-29–Arg-30) in the sequence of the *β* chain was confirmed by sequencing the first three steps of the CNBr-generated fragment CB1 (Fig. 3). The sequences of peptides T3 and T12 in the *β* chain (eluting in the same peak in RP-HPLC in Fig. 2) were obtained after their separation in a second purification step on RP-HPLC.

**Functional Characterization**—A large Bohr effect is observed in eel anodic Hb (Fig. 4). The Bohr factor ($\phi = 5 \log P_{50} / \log pH$, which represents the average number of protons bound upon heme oxygenation) at pH 7.5 is ~0.27 in the stripped hemoglobin and ~1.85 in the presence of GTP, the major erythrocytic phosphate in eel (4). This indicates that at pH 7.5 the number of oxygen-linked protons bound by tetrameric hemoglobin in the deoxygenated state can be increased by GTP from 1.08 (in the stripped hemoglobin) to 7.4. The Bohr effects measured in the absence and in the presence of GTP can be superimposed by a simple shift of the pH scale, indicating that the effect of GTP is essentially to increase the pK$_a$ values of the Bohr groups. The decrease in saturation at low pH in the presence of GTP and 1 atm of oxygen indicates the presence of the Root effect (Fig. 4, inset). Accordingly, the Hill coefficient $n_{50}$ falls to approxi-

![Fig. 3. Amino acid sequence of the *α* and *β* chains of the anodic Hb of the eel.](image)

![Fig. 4. Bohr effect (pH dependence of oxygen tensions and cooperativity at half-saturation, $P_{50}$ and $n_{50}$) of the anodic eel Hb measured at 20 °C in 0.1 M HEPES buffer in the absence and presence of 0.1 M KCl or 1 mM GTP.](image)
low pH with GTP (Fig. 4). The agreement between the data obtained with the two techniques, moreover, indicates that small concentrations of the enzymatic reducing system (present only in tonometrical studies) does not influence the oxygen binding properties of hemoglobin. A weak effect of KCl on the oxygen affinity is illustrated in both tonometrical and diffusion chamber experiments (Fig. 4).

Extended Hill plots for oxygen equilibrium experiments at different pH values and in the absence and presence of GTP are shown in Fig. 5, where deviations from linearity of the lower asymptote reflecting cooperative interactions in the T state are evident. A unitary slope in the upper asymptote indicates noncooperative binding in the R state. The equilibrium data are satisfactorily described by the co-operon model, as indicated by the curves obtained by fitting Equation 5 to the data (Fig. 5).

The allosteric parameters obtained in the fitting procedures are reported in Table I. The parameter \( i \) describes the overall or the apparent cooperative interactions within the T state as positive \((i > 1)\) or negative \((i < 1)\). Reliable estimates for \( K_R \) are in general difficult to obtain (in particular, in a Root-effect hemoglobin) as they require several data points at saturations \( \approx 99\% \). The \( K_R \) value reported in Table I is thus the mean value \((\approx 10\%)\) calculated for the stripped hemoglobin at pH 7.935 and held fixed to fit the allosteric constants in the other data sets as described (37). No significant variations were observed in, and regardless of, the values of the other parameters. Partly because of the high number of parameters fitted, a large uncertainty was found in the determination of \( L \) and \( i \), but not in \( K_T \). Compensating variations of \( L \) and \( i \) are presumably implied in nonconvergence in three data sets (Table I).

As illustrated in Table I, increasing proton concentration stabilizes the T relative to the R state \( (L \text{ increases}) \) and decreases \( K_T \) (as generally is observed in vertebrate hemoglobin) in the stripped hemoglobin. In the presence of GTP, \( K_T \) does not decrease further below pH 7.48 where the apparent cooperativity in the T state becomes negative as \( i \) decreases to values significantly below unity at low pH. Under these conditions, the hemoglobin molecule can be considered as locked in the low affinity conformation as indicated by the high values of \( L \). In the absence of cofactors, \( i \) remains above unity in the pH range investigated.

**DISCUSSION**

A remarkable feature of the oxygen equilibrium curves for the anodic Hb of eel is the deviation from linearity of the lower asymptote, which reflects the presence of cooperative interactions within the T state. Such behavior has not been reported before in other fish hemoglobins where such low oxygen saturation levels were not analyzed. In T state crystals of human HbA, a small amount of cooperativity compensates for inequivalent binding to the \( \alpha \) and \( \beta \) subunits, resulting in perfectly noncooperative \((i = 1)\) oxygen binding (18, 19). This fits neatly with the unitary slope of the lower asymptote observed in an extended Hill plot of human HbA in solution, which allows the use of the two-state MWC model for analysis of the allosteric properties. In the anodic eel Hb as well as in other Root-effect hemoglobins (38, 39), highly biphasic oxygen binding curves with apparent negative cooperativity are observed at low pH and in the presence of organic phosphate (Fig. 5). These particular oxygen binding properties together with the nonlinear lower asymptote cannot be described by the MWC model. We show that the functional properties of eel anodic Hb can be described by assuming the presence of cooperative interactions in the T state, as included in the co-operon model (25, 26). In its original formulation, the model assumes that the hemoglobin tetramer consists of two independent \( \alpha \beta \) dimers, each representing a cooperative unit or co-operon. However, later studies have shown that the two \( \alpha \beta \) dimers \((\alpha_1 \beta_1 \text{ and } \alpha_2 \beta_2)\) cannot be considered as functionally unrelated but that in the T state, ligation at one subunit enhances ligation at the other subunit of the same dimer and inhibits ligation at the opposite dimer (22). In our study, the parameter \( i \) therefore includes not only the intradimer cooperativity as in the original model but also any functional interaction between dimers across the \( \alpha_1 \beta_2 \) interface. Moreover, the situation in Root-effect hemoglobins is complicated by a large functional heterogeneity of the chains in the T state (40, 41), which is responsible for biphasic oxygen equilibrium curves at low pH and with organic phosphates and would contribute negatively to the T state cooperativity and decrease the value of \( i \). This would explain both the low values of \( i \) (Table I) and \( n_{50} \) (Fig. 4) found under these conditions, where the tetrameric molecule is essentially in the T state. It is important to note that the effect of organic phosphates is to shift the allosteric equilibrium toward the T state and to increase the pK, of Bohr groups (Fig. 4) rather than to enhance functional subunit heterogeneity in itself (41), in agreement with the conclusion that organic phosphates and protons have a similar allosteric effect (42). The
apparent negative cooperativity between the $\alpha$ and $\beta$ chains found at low pH with GTP therefore reflects a larger pH dependence (or a larger tertiary Bohr effect) of one of the two chains in the T state. By this mechanism, eel anodic Hb may release oxygen even in the absence of the T $\rightarrow$ R quaternary transition, which is the basis for the large Bohr effect observed in Root-effect hemoglobins. The increase in $L$ and in the functional subunit heterogeneity at low pH is the basis for the larger decrease in oxygen saturation at high oxygen pressures found in Root-effect hemoglobins. In human HbA, a pH decrease produces an increase in $L$ and a decrease in $K_T$ (32) so that the decrease in oxygen saturation is larger at low oxygen tension. These two different mechanisms may relate to the different physiological roles of the two pH effects. The Bohr effect enhances the amount of oxygen released in the metabolizing tissues (at low oxygen tension), and the Root effect allows release of a large amount of oxygen in the eye and swim bladder (at high oxygen tensions (38)).

The inverse relationship between $L$ and $i$ suggests that in the T state of eel anodic Hb, positive cooperative interactions prevail in the presence of the T $\rightarrow$ R transition, whereas negative interactions become apparent in the absence of the quaternary transition. Although a detailed analysis of the opposing factors contributing to heme-heme interactions is impossible at this stage, it appears that at the level of the $\alpha_1\beta_2$ interface, cooperative interactions in the T state may either be negative (and produce the T $\rightarrow$ R transition, as in human HbA (22)) or positive, thereby allowing ligand binding to proceed through the T state. Thus, a fundamental difference between Root-effect hemoglobins and hemoglobins with a normal Bohr effect such as human HbA is that at low pH, Root-effect hemoglobins remain in the T state, indicating that the $\alpha_1\beta_2$ interface remains stable in the T state upon oxygenation, whereas human HbA switches to the R conformation. The comparison of the primary structure of the anodic hemoglobin of the eel with that of the cadioid Hb and other fish hemoglobins reveals that several concomitant factors may contribute to the expression of the Root effect in fish hemoglobins: a particular configuration of the $\alpha_1\beta_2$ and $\beta_1\beta_2$ interfaces and the presence of proton binding groups.

The $\alpha_1\beta_2$ Contact—Side-chain packing at this interface is likely to be the major reason for the larger rotation of the two dimers in the R state found in the hemoglobins of spot and $P$. bernacchii compared with human HbA. Moreover, a different $\alpha_1\beta_2$ interface in fish hemoglobins is consistent with the lower tendency to split into dimers than human HbA (43).

In human HbA, at the dovetailed $\alpha_1\beta_2$ switch contact (between the C helix and CD corner of the $\alpha$ subunit with the FG corner of the $\beta$ subunit), His-FG4 $\beta$ packs between the side chains of Pro-CD2a and Thr-C6a in the T state, passes over one helix turn during the T $\rightarrow$ R transition, and packs between Thr-C6a and Thr-C3a in the R state. The ability of Root-effect hemoglobins to remain in the T state even when ligated may be related to a switch region different from that of human HbA and other fish hemoglobins (Table II). On the $\alpha$ chain, Gln is present at position C3, Thr or Ala is present at C6, a small residue (Ser, Ala, Thr) replaces the bulky Pro at position CD2, and Trp is highly conserved at CD4 whereas His is conserved at position FG4 of the $\beta$ chain. Moreover, fish hemoglobins possess an additional residue in position CD5 of the $\alpha$ chain compared with human HbA (Table II). Ligation of $\beta$ subunits within the T state of human HbA results in profound steric hindrance between the side chains of His-FG4$\beta$ and Pro-CD2a (44). The substitution of Pro-CD2a with a smaller and more flexible residue in Root-effect hemoglobins (Ser, Ala, or Thr; Table II) is likely to stabilize $\beta$ chain ligations in the T state. Ligation of $\alpha$ subunits in the quaternary T state may be favored by replacements at the flexible joint interface between the FG corner of the $\alpha$ chain and the C helix of the $\beta$ chain. In deoxy human HbA, Arg-FG4$\alpha$ is bound to Glu CD2$\beta$, which is replaced by a neutral amino acid residue in Root-effect hemoglobins (Ser, Ala or Gly; Table II), so that Arg-FG4$\alpha$ may instead form a hydrogen bond with Gln-C5$\beta$ (conserved in all fish hemoglobins) as found in deoxy trout ($Oncorhyncus mykiss$) HbI (45). The same interaction between Arg-FG4 and Gln-C5$\beta$ is present in oxygenated crystals of T state human HbA (20), which indicates that the replacement of Glu-CD2$\beta$ may stabilize intermediates in the oxygenation process of T state molecules (45). The location of the groups at the switch contact and at the flexible joint that may stabilize ligand binding in the T state is shown in Fig. 6.

Hemoglobin molecules that remain in the low affinity T state at low pH even when ligated will also have a larger number of oxygen-linked protons or a larger Bohr factor than hemoglobins that switch to the R state upon ligation. This means that the number of proton-binding sites in Root-effect hemoglobins does not need to be higher than in human HbA. The stabilization of the T state in Root-effect hemoglobins may be achieved not by an increased number of salt bridges but by a different allosteric mechanism where the stabilization of the $\alpha_1\beta_2$ interface upon oxygenation is an essential condition.

**Proton-binding Sites—** Anodic eel Hb can bind seven to eight Bohr protons per tetramer in the presence of GTP at physiological pH. A major candidate as a proton-binding site is His-HC3$\beta$, which is conserved in all Root-effect hemoglobins (Table II). Glu-FG1$\beta$ is also generally conserved in Root-effect hemoglobins (except for one of the three Root-effect hemoglobins of the antarctic *Pleuragramma antarcticum* where it is substituted by Gln (46)), indicating that a salt bridge between these two residues may be formed in the T state, as known for human HbA (where Asp is in position FG1$\beta$). Quite unexpectedly, in the crystal structure of the deoxygenated *P. bernacchii* Hb1...
Table II

Conservation of functionally important residues in Root-effect hemoglobins in comparison with non-Root-effect hemoglobins

Amino acid sequences are from the Swiss data protein bank except for P. antarcticum (46), T. newnesi (15), and A. mitopteryx (52).

| Chain | α Chain | β Chain |
|-------|---------|---------|
|       | C3      | C6      | CD2 | CD4 | CD5 | NA2 | NA3 | CD2 | EPF6 | F9 | FG1 | FG4 | H21 | HC3 |
| Root-effect Hb |         |         |     |     |     |     |     |     |     |     |     |     |     |     |
| A. anguilla anodic Hb | Gln | Ala | Ala | Trp | Lys | Glu | Trp | Ala | Lys | Ser | Glu | His | Arg | His |
| O. mykiss HbIV | Glu | Ala | Ser | Trp | Ala | Glu | Trp | Ser | Lys | Ser | Glu | His | Arg | His |
| Cyprinus carpio | Glu | Thr | Ala | Trp | Ala | Glu | Trp | Ala | Lys | Ser | Glu | His | Arg | His |
| N. angustata Hb1 | Glu | Thr | Ser | Trp | Pro | Lys | Trp | Ser | Ala | Ser | Glu | His | Lys | His |
| Chelodonichthys kumu | Glu | Thr | Thr | Ser | Trp | Thr | Glu | Trp | Ala | Lys | Ser | Glu | His | Arg | His |
| P. bernacchii Hb1 | Glu | Thr | Ser | Trp | Pro | Lys | Trp | Ser | Ala | Ser | Glu | His | Lys | His |
| P. antarcticum Hb1 | Glu | Thr | Ser | Trp | Pro | Gly | Trp | Gly | Ala | Ser | Glu | His | Lys | His |
| Non-Root-effect Hb |         |         |     |     |     |     |     |     |     |     |     |     |     |     |
| Human | Thr | Thr | Pro | Phe | His | Leu | Glu | Lys | Cys | Asp | His | His | His |
| Lepidosiren paradoxus | Gly | Ser | Pro | Phe | Gly | His | Trp | Asn | Lys | Ser | Glu | His | Arg | His |
| Latimeria chalumnae | Gln | Val | Asp | Phe | Thr | His | Trp | Lys | Lys | Phe | His | His | Arg | His |
| Electrophorus electricus | Gln | Thr | Ala | Trp | Ser | Glu | Leu | Ala | Lys | Ser | Glu | His | Lys | His |
| G. acuticeps | Gln | Ile | Ser | Trp | Pro | Asn | Trp | Ser | Ala | Ser | Glu | His | Lys | His |
| T. newnesi Hb1 | Gln | Ile | Ser | Trp | Pro | Gly | Trp | Gly | Ala | Ser | Glu | His | Lys | Val |
| A. mitopteryx | Gln | Ile | Asn | Trp | Pro | Gly | Trp | Gly | Ala | Ser | Glu | His | Lys | His |
| O. mykiss Hb1 | Gln | Thr | Ser | Trp | Ala | Glu | Trp | Gly | Lys | Ala | Asn | Phe | Ser | Phe |
| A. anguilla cathodic Hb | Ala | Val | Ser | Trp | Pro | Lys | Trp | Ser | Ala | Ser | Glu | His | Lys | His |

Fig. 6. Schematic representation of the deoxygenated structure of a Root-effect hemoglobin (P. bernacchii Hb1 is from the Brookhaven National Laboratory Protein Data Bank, file 1HBH), where the residues GluC338a, Thr-C341a, AspC324a, SerC324b, SerC325b, and His-FG497β at the α2β2 and α2β1 interfaces are indicated. The notations A, B, C, and D at the residues refer to the subunits α, β1, α2, and β2, respectively.

(47), His-HC3β was found free in solution and not bound to Glu-FG1β, which leaves a major part of the Bohr and Root effects difficult to explain. Asp-G1α and Asp-G3β were indicated as possible Bohr groups in this hemoglobin, as they move closer to each other upon deoxygenation and could thereby increase their pKα and share a proton. By analogy with P. bernacchii, Hb1, Asp-G1α, and Asp-G3β have been proposed as binding sites for two of the four oxygen-linked protons in spot Hb, where the remaining two protons would bind between the N terminus and His-HC3β of each β subunit (13). However, the contribution to the Bohr effect of these two Asp residues appears questionable since they are conserved in all fish hemoglobins including trout HbI, which exhibits pH-independent oxygen binding. Moreover, in the deoxygenated form of trout HbI, Asp-G3β makes a salt bridge with Arg-G6β (conserved in fish hemoglobins or replaced by Lys in antarctic teleosts), thus preventing the two Asp residues from increasing their pKα and participating in the Bohr effect (45).

We propose that another major Bohr group in eel anodic Hb and other Root-effect hemoglobin may be His-FG4β, located at the switch interface. Histidines in both positions HC3β and FG4β are replaced in the cathodic hemoglobins of eel (5), trout (48), and moray (49), all showing pH-independent oxygen binding or a weak reverse Bohr effect. In human HbA where the C-terminal His of the β chain has been cleaved, His-FG4β contributes to the Bohr effect under specific conditions of ionic strength and pH (50). This residue packs against different side chains of the C helix and CD corner of the α chain in the T and the R state. Proton uptake by His-FG4β upon deoxygenation would be favored by a pKα increase in the T state (by interaction with polar or negatively charged groups, including the C-terminal end of the C helix of the α chain (50)) or by a pKα decrease in the R state (e.g., by a more hydrophobic environment of this His residue in the R than in the T state). An Ile residue at position C66 in the hemoglobins of T. newnesi (15), Gymnodraco acuticeps (51), and Aethotaxis mitopteryx (Ref. 52 and Table II) may not be able to provide the favorable environment for oxygen-linked protonation of His-FG4β, which is consistent with the lack of a Root effect in these hemoglobins. The highly cooperative oxygen binding of these hemoglobins, even at low pH values (pKa > 2), indicates that the allosteric T → R transition is fully operative or, in other words, that the switch interface in the T state is not stable upon oxygenation. Other replacements at the C helix of the α chain in the presence of His-FG4β agree well with the absence of the Root effect (Table II).

Proton Binding at the ββ1 Interface—GTP binds in the central cavity between the two β chains in the T state. The binding site for organic phosphates in fish hemoglobins involves the N terminal, Asp- or Glu-NA2, Lys-EPF6, and Arg-H21 (53). Since GTP increases the pKα of Bohr groups, the remaining oxygen-linked proton-binding sites in anodic eel hemoglobin thus appear to be localized in the central cavity, and the most likely candidates appear to be the N terminus of the β chain and Lys-EPF6β, given the high pKα of the Arg side chain. The excess of positive charges at the ββ1 interface represents a destabilizing factor for the T state of human HbA, which, in the absence of anions, results in an increased oxygen affinity due to the shift toward the high affinity state (54). The pKα of the N terminus and Lys-EPF6β in the T state may therefore be lowered by adjacent positive charges (e.g., Arg-H21β) to values between 6.0 and 8.0, where the Bohr effect is observed. Moreover, in Root-effect hemoglobins, a narrower central cavity in the R state than in human HbA would further reduce the pKα of these groups (13). By this mechanism, the groups in the central cavity attain an increased proton affinity in the T state.
so that they can contribute to the alkaline Bohr effect. Replacement of Lys-EF6β by Ala in the hemoglobins of *P. bernacchii*, *P. antarcticum*, and *N. angustata* may be compensated by the substitution of Arg-H21β with Lys (Table II) that has a lower pKₐ. Moreover, Lys replaces Glu-NA₂β in *N. angustata* Hb1, in agreement with a Bohr effect larger than in *P. bernacchii* Hb1 (55). In the absence of organic phosphates, the positively charged residues in the central cavity appear to act as reverse Bohr groups when the alkaline Bohr groups and the residues at the α₁β₂ are replaced, as proposed for the cathodic eel Hb (55).

This site is of primary importance for phosphate modulation of blood oxygen affinity. In the eel, under hypoxic conditions, oxygen affinity increases rapidly through a decrease in the intra-erythrocytic concentration of organic phosphates (56), particularly GTP, whereas the relative amount of the two hemoglobin components remains unaffected (4).

**Conclusion**—The present data on eel anodic Hb indicate that several regions of the tetrameric hemoglobin molecule contribute to the Root effect: 1) the α₁β₂ interface with Gln-C₆α, Thr-(or Ala)-C₆α, a small apolar or weakly polar residue (Ala, Ser, Thr) at CD2α, Trp-CD4α and His-FGβ at the switch contact, and a nonnegatively charged residue at CD2β (Ala, Gly, or Ser) at the flexible joint; 2) the β₁β₂ interface (as indicated by Mylvaganam *et al.* (13)), including the N terminus of the β chain, Lys-EF6α, Trp-NA₃β, and Arg-H21γ; and 3) His-HCβ₂. Substitutions in at least one of these regions correlate with the absence of the Root effect (Table II).

In the anodic eel Hb, the Bohr effect may be accounted for by proton binding at the N terminus, His-HC₃, His-FG₄, and Lys-EF₆β of the β chains. Protons appear to stabilize the α₁β₂ interface in the T quaternary state (even in the presence of oxygen) and destabilize the β₁β₂ interface in the R state, as proposed for spot Hb (13), thereby shifting the allosteric equilibrium to the Root effect: 1) the α₁β₂ interface to the Root effect: 1) the α₁β₂ interface (as indicated by Mylvaganam *et al.* (13)), including the N terminus of the β chain, Lys-EF6α, Trp-NA₃β, and Arg-H21γ; and 3) His-HCβ₂. Substitutions in at least one of these regions correlate with the absence of the Root effect (Table II).

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