The Structural and Functional Analysis of the Hemoglobin D Component from Chicken*

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Oxygen binding by chicken blood shows enhanced cooperativity at high levels of oxygen saturation. This implies that deoxy hemoglobin tetramers self-associate. The crystal structure of an R-state form of chicken hemoglobin D has been solved to 2.3-Å resolution using molecular replacement phases derived from human oxyhemoglobin. The model consists of an \( \alpha_b - \beta_b \) tetramer in the asymmetric unit and has been refined to a \( R \) of 0.222 (incomplete oxygen saturation resulted in spuriously high Hill coefficients) for 29,702 reflections between 10.0- and 2.3-Å resolution. Chicken Hb D differs most from human oxyhemoglobin in the AB and GH corners of the \( \alpha \) subunits and the EF corner of the \( \beta \) subunits. Reanalysis of published oxygen binding data for chicken Hbs shows that both chicken Hb A and Hb D possess enhanced cooperativity in vitro when inositol hexaphosphate is present. The electrostatic surface potential for a calculated model of chicken deoxy-Hb D tetramers shows a pronounced hydrophobic patch that involves parts of the D and E helices of the \( \beta \) subunits. This hydrophobic patch is a promising candidate for a tetramer-tetramer interface that could regulate oxygen binding via the distal histidine.

The oxygen binding curves of chicken blood samples taken at different stages of development exhibit Hill coefficients which exceed 4.0 (super-cooperativity) at oxygen saturation levels between 70 and 90% oxygenation (1–5). Elevated levels of oxygen-binding cooperativity should be advantageous because such hemoglobins (Hbs) would deliver a greater quantity of oxygen to the tissues under normal physiological conditions. Super-cooperativity has also been observed in the blood and Hbs of several species of amphibians (6–8), reptiles (9–10), other birds (1, 11), and embryonic mammals (12–14). Although incomplete oxygen saturation resulted in spuriously high Hill coefficients in much of the early data (see Refs. 15–18), the data of Lapennas and Reeves (5) on chicken blood and Holland et al. (12–14) on embryonic marsupial blood were obtained at saturating pressures of \( O_2 \).

The blood of adult chickens, as in most other birds, is composed of two Hb components that have identical \( \beta \) chains but differ in the sequences of the \( \alpha \) subunits. Hb A (\( \alpha_a - \beta_b \)) and Hb D (\( \alpha_D - \beta_b \)) are expressed in a 3:1 ratio in adult chickens (19). The sequence of the \( \alpha \) chain from Hb D (\( \alpha^D \)) is 58.9% identical to that of Hb A (\( \alpha^A \)). The large Hill coefficients observed in chicken blood imply that the deoxy-Hb tetramers associate into oligomers because the Hill coefficient cannot be greater than the number of subunits. Although super-cooperativity has not been reported for the purified adult chicken Hb components, the self-association of deoxy tetramers is known. Sedimentation analysis of chicken Hbs A and D showed that only deoxygenated Hb D self-associates. This analysis demonstrated that deoxy-Hb D forms a dimer of tetramers with an association constant of 1.26 \( \times \) \( 10^4 \) \( \text{m}^{-1} \) (20). Because the two chicken Hb components only differ in their \( \alpha \) chains, it was concluded that the tetramer-tetramer contacts lie on the surface of the \( \alpha_D \) subunit (20). This led to a proposed model that describes the interface that might form between two Hb D tetramers (20).

The cooperative binding of oxygen by tetrameric Hbs arises from the transition of the Hb from a conformation with a low oxygen affinity (T-state) to a conformation with a high oxygen affinity (R-state) (21, 22). Much of our understanding of this process has come from the crystallographic analysis of the unligated (T-state) and ligated (R-state) structures of both human Hb A (23–26) and horse Hb (27–30). Although a structural explanation of super-cooperativity requires the crystallographic analysis of both the R- and T-state forms of chicken Hb D, the analysis of intermediate structures are likely to be necessary for understanding the mechanism of cooperativity because knowledge of two thermodynamic states does not itself determine the pathway between them. We report here the structure of an R-state form of chicken Hb D solved with diffraction data to 2.3-Å resolution. This structure provides the first step towards a model for enhanced cooperativity and is the first structure reported for the minor Hb (D) component of birds, and the second structure of an avian Hb (31). The re-analysis of the oxygen binding data in the literature demonstrates a previously, overlooked relationship between super-cooperativity and inositol hexaphosphate (IHP) binding.

MATERIALS AND METHODS

Purification, Crystallization, and Data Collection—Chicken Hb D was purified from chicken blood using DEAE and gel filtration chromatography as described previously (20). Several grains of sodium dithionite (Miles-Platting, Manchester, United Kingdom) were added to the Hb solutions prior to crystallization. Conditions for crystallization were screened by the sparse matrix technique (32) with the hanging drop method (33). Chicken Hb D was crystallized with a
combination of PEG 3350 and either lithium sulfate or sodium acetate as the precipitant. The precipitant concentration was varied from 20 to 30% polyethylene glycol and from 10 to 30 mg/ml salt, with a protein concentration of 20 mg/ml in 50 ml Tris/HCl, pH 7.5. The crystal setups were prepared under nitrogen in a glove box (Bactron 1 Model Inc), which was equipped with a constant nitrogen flow. The crystals were transferred in liquid nitrogen to an insulated dewar that was flushed with nitrogen. The desiccator was then placed in a cold room at 4 °C for 2 weeks. Crystals of Hb D grew as plates of about 0.1 mm in thickness and 1.0-mm wide.

X-ray data were collected on a San Diego Multiwire area detector equipped with a Rigaku RU-200 rotating anode generator operated at 50 kV and 110 mA. The initial X-ray data indicated that the Hb D crystals belong to the space group P21 with cell dimensions of a = 53.96 Å, b = 80.51 Å, c = 82.11 Å, and β = 104.51°. Assuming the presence of two tetramers of 66,452 daltons in the unit cell, the Matthews' parameter is equal to 2.6 Å3/dalton. A data set was collected from nine orientations of a single crystal by the method described by Xueg et al. (34). The data were collected at room temperature, with a rotation angle of 0.12° in a, and an exposure time of 60 s. The measured intensities were processed with the University of California, San Diego package (35). The resulting data set is 91% complete to 2.1 Å resolution with an overall multiplicity of 6.8, and an overall Rmerge of 8.1%. Because of the low signal to noise in the last resolution shell, only the data to 2.3 Å resolution were used in the refinement of the Hb D model. The last three rounds of refinement used a 2.5–2.3 Å has an average multiplicity of 3.6, a Rmerge of 23.9%, and an average I/σ(I) of 2.3.

Structure Determination—The initial phases were obtained by the molecular replacement method with the human Hb tetramer serving as the search model. The α and β chains of chicken Hb D are 61.7 and 65.7% identical to those of human Hb A, respectively. Both the deoxy (26) and the oxy (25) forms of human Hb were used as search models because there was some doubt that the crystals of Hb D were deoxy. Each of the Hb models consisted of the human sequence with all identical residues being retained and all non-identical residues being truncated to alanine. All the molecular replacement and refinement routines were performed using the computer program X-PLOR version 3.1 (36) running on a DEC ALPHA server. The molecular replacement protocol included a Patterson correlation refinement step (37) that allowed each of the subunits of the search model to be optimized by rigid body refinement before the translation function was performed. The cross-rotation function followed by the Patterson correlation refinement using either Hb search model resulted in a rotation solution at 288°, 45°, 348° (θ1, θ2, θ3 of xxy Eulerian angles). The translation function for either model produced a clear peak at 0.407, 0.000, and 0.232 (xyz in fractional coordinates). An examination of the correctly positioned models indicated that the conformation of both models described an R-state Hb; therefore, only the oxy model, which gave a slightly better signal to noise ratio in the translation function (12.4 σ/mean compared with 10.1 σ/mean for the deoxy model) was refined against the observed data. The initial R-factor of the positioned model was 0.457 for all measured data between 8.0- and 3.0-Å resolution.

RESULTS

The Refined Model—Crystals of chicken Hb D belong to the monoclinic space group P21 with one αₐβ₃ tetramer (a total of 574 residues) in the asymmetric unit. The α₁ and β subunits of chicken Hb D are composed of 141 and 146 amino acid residues, respectively. The refined model of chicken Hb D consists of 570 amino acid residues, 4 heme groups, 141 water molecules, and three oxygen ligands. The last residue of the αₐ subunit (Arg141) and the last three residues from the β₁ subunit, Lys144(HC1), Tyr145(HC2), and His146(HC3) were omitted from the final model due to the lack of electron density. In other areas of poor electron density, the side chains of 4 residues were truncated to glycine, 25 were truncated to alanine, and 6 were truncated by two or more atoms. The R-factor of the refined model is 0.222 (R-free = 0.257) for all measured reflections between 10–2.3 Å resolution (Table I). The geometry of the chicken Hb D model is excellent when compared with the ideal parameters of Engh and Huber (39). The Ramachandran plot indicates that 89.2% of the non-glycine, non-proline dihedral angles lie in the most favored regions whereas 9.8 and 1% of the residues have ψ and φ angles which lie in the additionally allowed and generous regions, respectively. No residue has dihedral angles in the disallowed regions of the Ramachandran plot. The conformation of each residue with ψ-φ angles in the generous region of the Ramachandran plot was verified by

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fictitious, each of these residues into simulated annealing omit maps.

Noncrystallographic Symmetry—The presence of an αβ₁₆ tetramer in the crystallographic asymmetric unit results in the generation of a molecular 2-fold axis of NCS between the two αβ dimers. Differences between the two dimers are localized to small regions of the model which were not restrained by NCS. The first and last 4 residues from both subunits show the largest differences as indicated by average r.m.s. differences of 1.1 Å/Cα atom. The residues which form the EF corner of the α subunits (residues 75–77) show small differences as indicated by average r.m.s. differences of 0.35 Å/Cα atoms. The remaining Cα atoms have average r.m.s. differences of 0.05 Å/Cα atom due to the tight restraints. The side chain atoms show larger differences. Excluding the first four and last 4 residues from each subunit, the superposition of the α₁β₁ dimer onto the α₂β₂ dimer gives a r.m.s. difference of 0.47 Å for 2039 main chain and side chain atoms.

Overall Structure and the Comparison with Human Oxy-Hb A—Chicken Hb D has a quaternary structure which is consistent with other R-state Hbs, but differs from other T-state or R2 state structures. The superposition of 570 Cα atoms from chicken Hb D onto the corresponding Cα atoms of human oxy-Hb (25) gives an r.m.s. value of 1.0 Å. The superposition of the Ca atoms from chicken Hb D onto the corresponding atoms from either T-state or R2-state human Hb shows larger differences. The r.m.s. values are 2.4 and 1.7 Å for the superposition of 570 Cα atoms from chicken Hb D onto the Cα atoms from human T-state (26) and R2-state (53) Hbs, respectively.

As expected, the folds of the α and β subunits of Hb D are both similar to those of human Hb, with each α subunit composed of seven helical segments (labeled A; B, C, E, F, G, and H) and each β subunit composed of eight helical segments (labeled A–H). The inter-helical segments ("corners") formed between helical segments are labeled according to the nomenclature used for myoglobin. The superposition of 141 Cα atoms of α₁, 141 Cα atoms of α₂, 143 Cα atoms of β₁, and 146 Cα atoms of β₂ of human oxy-Hb (25) onto the corresponding subunits of Hb D gives r.m.s. differences of 1.1, 0.85, 0.79, and 0.78 Å, respectively (Figs. 1 and 2). Both subunits of Hb D show moderate differences in the positioning of the four NH2-terminal and four COOH-terminal residues. However, these segments in chicken Hb D also show the largest differences between NCS related subunits, reflecting the increased flexibility of the termini from each R-state Hb subunit. If these residues are excluded, 133 Cα atoms of the α subunit and 138 Cα atoms of the β subunit from human Hb superimpose upon their counterparts in chicken Hb D with r.m.s. differences of 0.83 and 0.69 Å, respectively.

Several other regions of the α and β subunits from chicken oxy-Hb D also differ from the corresponding regions in human oxy-Hb. The AB corner of the α subunits of Hb D forms a more helical turn than its counterpart in human Hb (Fig. 1). This difference is due in part to the substitution of 2 glycine residues in human Hb with Ala^{18}(A16) and Glu^{229}(B3) in chicken Hb D. The tightness of this turn results in a slight change in the orientation of helix B in the α subunit of chicken Hb D. The replacement of a Pro^{114}(GH2) in human Hb with Gly^{114} in chicken Hb D results in a movement of the bulge located in the middle of the GH corner. In chicken Hb D, Gly^{114} is oriented with a φ angle of 42° and a ψ angle of −131°, causing a change in the orientation of this bulge, shifting the GH corner, moving the Cα atom of Lys^{115}(GH3) by 3.7 Å, and positioning its side chain so that it is rotated by 90° relative to that in human oxy-Hb. A third difference between the two Hbs involves the location of the COOH-terminal end of the E helices and of the EF corners (residues 73–84) of the β subunits so that the EF corner of the β subunit from Hb D is closer to its α helix (Fig. 2).

Comparison with Hb A of Bar-headed Goose—The structure of one other avian Hb (Bar-headed goose Hb A) has been reported, also in the R-state (31). Although the β chains of the two avian adult Hbs are very similar (95.2% sequence identity), chicken αD is only 56.0% identical to bar-headed goose αA, less than the 61.7% identity which exists between human α and chicken αD. The tertiary structures of the α and β chains from the two avian Hbs reflect the relative degree of sequence identity. The superposition of 143 Cα β-subunit atoms from chicken Hb D onto the corresponding atoms of the bar-headed goose Hb gives an r.m.s. difference of 0.30 Å, compared with an r.m.s. difference of 0.7 Å for the superposition of 140 Cα atoms in the α subunits. The α subunits of both bar-headed goose and chicken Hbs have nearly identical AB corners but differ in the GH corners. The analysis of other avian α chain sequences suggests that the residues that comprise the AB corner in both bar-headed goose and chicken Hbs are not absolutely conserved in other avian Hbs. Although an acidic residue is present at position 22 (B3) in 38 of the 40 avian α chains surveyed, only 15 of these avian Hbs retain a glycine at position 18 (A16). Avian Hbs with α subunits that retain a glycine only at position 18 would be expected to have an AB corner that differs from that of chicken Hb D. The αD subunits of all but one avian Hb D include a glycine at position 114 (GH2) whereas all 20 of the avian αA chains surveyed have GH corners with a proline at this position. This result suggests that the conformation of the GH corner observed in chicken Hb D will be characteristic of most Hb D components of avian blood.

The αβ₁ Interface—The αβ₁ interface is similar to that found in other vertebrate Hbs. Out of the 36 residues that form the αβ₁ interface in chicken Hb D (based on those residues with an atom which is within 4.0 Å of an atom from a neighboring subunit), 21 (58%) are identical to their counterparts in human Hb compared with 29 identities (81%) in bar-headed goose Hb A. Although most of these substitutions should not alter the stability of the αβ₁ interface, there are three substitutions, Gly^{114}(GH2), Glu^{103}(G10), and Ser^{119}(GH2), found in chicken Hb D which deserve comment. The replacement of a proline by a glycine at α114 in chicken Hb D results in the reorientation of the α114 main chain torsion angle, thereby

| Table I: The refinement statistics of the current model of chicken oxy-Hb D |
|---------------------------------------------|
| Resolution range                        | 10.0–2.3 Å |
| Number of reflections                   | 29702     |
| Number of non-hydrogen protein atoms    | 4329      |
| Number of Cα atoms                      | 172       |
| Number of O₂ ligand atoms               | 6         |
| Number of non-hydrogen water atoms      | 141       |
| R-factor                                 | 0.222     |
| R-free                                   | 0.257     |
| Rms deviation from ideal values          |           |
| Bond lengths                             | 0.008 Å   |
| Bond angles                              | 1.6 °     |
| Dihedral angles                          | 21.4 °    |
| Improper angles                          | 1.6 °     |
| Mean B-factors                           |           |
| Main chain atoms                         | 29.0 Å²   |
| Side chain atoms                         | 33.4 Å²   |
| Heme atoms                               | 23.2 Å²   |
| O₂ ligand atoms                          | 29.9 Å²   |
| Water atoms                              | 45.5 Å²   |
eliminating an intersubunit hydrogen bond. In place of this H-bond, the \( \alpha_1 \beta_1 \) interface is stabilized by two additional hydrogen bonds, one formed between the hydroxyl group of Ser\textsuperscript{b119}(GH2) and the carbonyl oxygen of Val\textsuperscript{a111}(G18) and a second between the side chains of Gln\textsuperscript{a103}(G10) and Asp\textsuperscript{b108}(G10). The addition of an extra H-bond should stabilize both the R- and T-states of chicken Hb D. Gln\textsuperscript{a103} is conserved in all avian \( \alpha \) sequences and about half of the avian \( \alpha \) sequences whereas Asp\textsuperscript{b108} is conserved in all 20 \( \beta \) sequences surveyed. Ser\textsuperscript{b119} is only present in four of the avian \( \beta \) sequences.

The \( \alpha_1 \beta_2 \) Interface—In human and horse Hbs, the \( \alpha_1 \beta_2 \) interface involves a total of 17 residues, all of which are highly conserved in vertebrate Hbs. During the T- to R-state transition, this interface acts as a switch. Consequently, both the R- and T-state conformations have distinct sets of interactions which contribute to the stabilization of the \( \alpha_1 \beta_2 \) interface. Like most R-state Hbs, the \( \alpha_1 \beta_2 \) interface of chicken Hb D includes a hydrogen bond formed between Asp\textsuperscript{a94}(G1) and Asn\textsuperscript{b102}(G4) and lacks a hydrogen bond between Asn\textsuperscript{a97}(G4) and Asp\textsuperscript{b99}(G1). Like the bar-headed goose Hb A, the \( \alpha_1 \beta_2 \) interface of chicken Hb D substitutes Gln\textsuperscript{a38}(C3) in place of a threonine found in mammalian Hbs. The hydroxyl group of Thr\textsuperscript{a38} in horse and human Hbs forms a hydrogen bond with the main chain carbonyl oxygen of His\textsuperscript{a297}(FG4) when the Hb has an R-state conformation. The side chain of Gln\textsuperscript{a38} in chicken Hb D cannot form an analogous hydrogen bond, thereby reducing the stability of the \( \alpha_1 \beta_2 \) interface. This substitution should increase the oxygen affinity of R-state chicken Hb D because the loss of a hydrogen bond would result in an increase in the concentration of \( \alpha \beta \) dimers which have high oxygen affinity. It is likely that Gln\textsuperscript{a38} would decrease the cooperativity of chicken Hb D. Samples of purified chicken Hb D exhibit Hill coefficients that are less than two in the absence of all organic phosphates (54).

The Heme Regions—The heme irons in chicken Hb D appear to be coordinated to either water or oxygen. Initially, it was assumed that the R-state model represented the aquomet form of Hb D and water molecules were modeled into the electron density located near the heme iron from each Hb subunit. Following four rounds of refinement and model fitting, each water moved to a position that is 2.9 Å away from the iron, allowing the water to hydrogen bond to the distal histidine. Although this distance is consistent with that observed in the structure of the aquomet form of \textit{Chironomous thummi} Hb (55), the water-to-iron distance of chicken Hb D differs from the corresponding values observed both in the structure of horse aquomet Hb (30) and in the high resolution structures of ferric porphyrins complexed with water ligands (56, 57). Re-examination of the electron density from a set of simulated annealing \( 2F_o - F \) omit maps suggests that three of the four subunits are ligated to oxygen and the fourth (the \( \alpha_2 \) subunit) is ligated to water (Fig. 3). An additional round of refinement allowed the O-1 atoms of the oxygen ligands of the \( \beta \) subunits to move to 1.8 Å from the heme iron and the oxygen ligand of the \( \alpha_1 \) subunit...
to refine to 1.7 Å from the heme iron. The orientation of the oxygen ligands from the two subunits differ. The Fe-O1-O2 angles for the α1 and α2 subunits are 138° and 173°, respectively. The water ligand of the α2 subunit refined to 1.8 Å from the heme iron, shorter than the 2.0 Å values observed in horse aquomet Hb (30). It would be unusual for the two α subunits to have different ligands. The ligand assignments based on the electron density at 2.3-Å resolution must be considered as tentative. Nevertheless, this model provides the best fit to the simulated annealing omit map (Fig. 3).

The heme groups of the α and β subunits of chicken Hb D resemble those of the ligated forms of other vertebrate Hbs (Table II) in that the iron atoms all lie near the plane of the heme atoms (defined by all heme atoms excluding those in non-planar side chains). The iron atoms of each subunit are approximately 0.2 Å out of the heme plane toward the proximal histidine. These values can be contrasted to the corresponding distances observed in human deoxy-Hb. In the absence of a ligand, the hemes of deoxy-Hbs should have iron-to-heme plane distances between 0.5 and 0.6 Å. The heme groups of chicken Hb D, as in all known Hbs, are ligated to the Ne-2 of the proximal histidine from each subunit (His87 in the α subunits and His92 in the β subunits). Each heme fits into a pocket lined with several hydrophobic residues. In chicken Hb D, the heme pockets of the α subunits are formed from 17 residues and those of the β subunits are formed from 16 residues. All of the

![Image](image-url)

**Fig. 3. The heme pockets for the α1 (top) and α2 (bottom) subunits.** The hemoglobin model is superimposed upon the electron density as calculated from a α1 weighted (75), simulated annealing omit 2Fo- Fc map in which both of the α heme groups were omitted from the map calculation. The α1 heme appears to be ligated to an oxygen molecule and the α2 heme is best fit with a water as the ligand.

**Table II**

|                | Oxy form | Deoxy form of human Hb A |
|----------------|----------|--------------------------|
|                | Chicken Hb D | Goose Hb A | Human Hb A |                               |
| Fe-Np          | 2.1 Å     | 2.1 Å             | 1.9 Å     | 2.1 Å      | 2.0 Å | 2.2 Å |
| Fe-Np avg      | 2.0 Å     | 2.0 Å             | 2.0 Å     | 2.0 Å     | 2.0 Å | 2.1 Å |
| Fe-H-plane     | 0.2 Å     | 0.2 Å             | 0.2 Å     | 0.2 Å     | 0.1 Å | 0.1 Å |
| Fe-H-plane avg | 0.2 Å     | 0.2 Å             | 0.3 Å     | 0.2 Å     | 0.2 Å | 0.1 Å |
| Fe-O           | 1.7 Å     | 1.9 Å             | 1.8 Å     | 1.8 Å     | 1.7 Å | 1.9 Å |
| FeO-1-O-2 angle| 148°      | 155°             | 170°      | 158°      | 152° | 158° |
| Fe B-factor    | 29 Å      | 19 Å              | 24 Å      | 19 Å      | 27 Å | 37 Å |
| O1 B-factor    | 22 Å      | 24 Å              | 24 Å      | 19 Å      | 18 Å | 42 Å |
| O2 B-factor    | 32 Å      | 39 Å              | 44 Å      | 29 Å      | 34 Å | 37 Å |
| Heme B-factor  | 24 Å      | 23 Å              | 25 Å      | 23 Å      | 33 Å | 39 Å |

|                |                               |                               |                               |                               |                               |
|----------------|                               |                               |                               |                               |                               |
| a              |                               |                               |                               |                               |                               |
| Np, pyrrole nitrogens of the heme.|
| b              | N-plane, plane of the heme defined by the four pyrrole nitrogens.|
| c              | H-plane, plane of the heme defined by all the heme atoms excluding those beyond the second atom of each side chain.|
| d              | This value is calculated from just the α1 subunit.|

For comparison, the geometric parameters of the unliganded heme from the structure of human deoxy-Hb (26) are also included. Unless specified, the values reported for chicken oxy-Hb D, goose oxy-Hb A, and human deoxy-Hb represent the mean value calculated from the two αβ dimers present in the asymmetric unit.
residues that contact the heme groups in the α subunits are conserved in human Hb. The only difference between the β heme pockets of chicken and human Hbs is the substitution of Serb70(E14) in chickens with Alab70 in humans. This difference is probably not significant because serine is found at this position in the β subunits of other mammalian Hbs. Thus the heme pockets of chicken Hb D show no differences from other vertebrate Hbs which could account for the super-cooperativity of chicken blood.

**DISCUSSION**

**Inositol Hexaphosphate-binding Site**—Organic phosphates lower the oxygen affinity of vertebrate Hbs by preferentially binding to the T-state of the tetramer (58, 59). The major organic phosphate in human red blood cells is bisphosphoglycerate, whereas that in chicken erythrocytes is inositol pentaphosphate (60). Most in vitro studies utilize the more readily available IHP. Both human and chicken Hbs bind IHP in a maximum ratio of 1 molecule of IHP per Hb tetramer (61). The crystallographic analysis of human Hb complexed with IHP indicates that IHP binds in a surface cavity formed between the two β subunits, the same site which also binds bisphosphoglycerate (62). In this crystal structure, the IHP-binding site includes the amino terminus of Valb1(NA1) as well as the side chains of Hisb22(NA2), Lysb82(EF7), Asnb139(H17), and Hisb143(H21) from both β subunits (62).

Chicken Hbs A and D bind organic phosphates more tightly than do mammalian Hbs. The oxy and deoxy forms of human Hb at pH 6.8 bind bisphosphoglycerate with association constants of $1.2 \times 10^3 \text{ M}^{-1}$ and $1.7 \times 10^4 \text{ M}^{-1}$, respectively (63). The CO R-state of chicken Hb A at pH 7.0 binds bisphosphoglycerate with an association constant of $1 \times 10^4 \text{ M}^{-1}$ (61), which is approximately the same as human deoxy-Hb. Similarly, the oxy form of a related avian Hb (Hb A from bar-headed goose) binds IHP with an affinity similar to that of human deoxy-Hb (64). Both the R- and T-states of chicken Hb bind IHP more tightly than the equivalent forms of human Hb, although the IHP binding constants were reported to be too large to be measured (61). The increase in the organic phosphate affinity of chicken and bar-headed goose Hbs over human Hb may be attributed to the substitution of 3 basic residues found in the β subunits of most avian Hbs. The replacement of Alab135(H13), Asnb139(H17), and Hisb143(H21) in human Hb with Argb135, Hisb139, and Argb143 in chicken Hb increases the positive charge of the phosphate binding cavity (62).

The organic phosphate-binding site of the R-state of chicken Hb D does not allow IHP to fit easily into its normal binding site. A reasonable model of chicken oxy-Hb with IHP can be made by moving the organic phosphate 4.5 Å toward the Hb surface, enabling IHP to interact with the amino terminus as well as the side chains of Lysh82 and Argb143 from one β subunit and the side chains of Lysh139 and Argb143 from the second β subunit (Fig. 4A). The R- to T-state transition opens up this cavity in most vertebrate Hbs. A deoxy model of chicken Hb D based on human Hb-IHP permits IHP to enter the phosphate-binding cavity. This position
that IHP binding is linked to the super-cooperativity observed in the presence of IHP (kindly provided by Dr. R. E. Isaacks. This particular data set was collected using the continuous recording method of Long-muir and Chow (65) with the modifications described by Lian et al. (66). Hbs A and D were purified by anion exchange chromatography. The oxygen-binding curves for Hbs A and D were measured in both the presence and absence of 2 mM IHP (54) at a Hb concentration of 1.7 mg/ml.

The original analysis of Isaacks et al. (54) reported Hill coefficients at 50% saturation. However, the functionally relevant values in Hbs which self-associate occur at higher oxygenation levels (67). Therefore, the O2-binding curves of chicken Hbs D and A were re-evaluated. The Hill coefficients were calculated as a function of oxygen saturation. Separate least-squares lines were fitted to the low oxygen saturation (<55% saturation) and high oxygen saturation (≥55% saturation) points from each curve (Fig. 5). The slope of the line gives n, the Hill coefficient. In all oxygen binding curves of Hb D, the lower part of the curve is described by Hill coefficients that vary between 1.5 and 1.7. The Hill coefficient of the upper part of its O2-binding curve is also about 1.8 in the absence of all organic phosphate effectors (Hb D alone). The addition of 2 mM IHP to Hb D increases the Hill coefficient of the upper half of the oxygen-binding curve to 4.2 (Fig. 5A), demonstrating that super-cooperativity does occur in vitro. Unexpectedly, the Hill plots of chicken Hb A behaved similarly. The upper half of Hill plots for the oxygen binding of Hb A showed elevated levels of cooperativity in the presence of 2 mM IHP whereas the lower half of the plot showed Hill coefficients of 1.6 (Fig. 5B). The lack of super-cooperativity observed for chicken Hb D in the absence of IHP is probably due to the low (102 m\(^3\) m\(^{-1}\)) concentrations of Hb used in these experiments. Super-cooperativity of chicken Hb D would not be observed at this concentration because over 69% of deoxy-Hb D would exist as tetramers given the tetramer to octamer association constant. Measurement by sedimentation velocity measurements (20). However, over 92% of deoxy-Hb D would associate into octamers at Hb concentrations (~300 mg/ml) normally found in chicken red blood cells. The observation of enhanced cooperativity in chicken Hb samples with IHP suggests that this process is linked to the binding of inositol pentaphosphate in vivo. This result also implies that IHP aids the formation of (\(\alpha\text{2}\beta\text{2}\))2 octamers. The increased stability of the tetramer-tetramer interaction would allow the (\(\alpha\beta\))4 to be maintained in partially ligated structures, thereby promoting super-cooperativity. Additional work will be required to measure the effect of IHP on the tetramer to octamer association constant.

It should be mentioned that the oxygen-binding measurements of Isaacks et al. (54) suffer from two possible sources of error. The first possible source of error is due to the assumption that both the production of oxygen by the electrode and the
consumption of oxygen by beef heart mitochondria are linear processes. The original analysis of Longmuir and Chow (65) demonstrated that both of these assumptions were true in the absence of organic phosphates. However, it is not known if both the oxygen production and oxygen consumption processes of this experiment are truly linear when IHP is added to the sample. The second source of possible error is due to an incomplete saturation of the Hb sample. It has been demonstrated that artificially high Hill coefficients occur when the 100% saturation point is underestimated (15–17). This problem is particularly applicable to chicken Hbs because of their low oxygen affinity. The 100% saturation point for the oxygen binding analysis of Isaacks et al. (54) was estimated with oxygen saturation points measured at relatively high oxygen pressures (~265 torr). The 100% saturation point of this analysis can be underestimated by values between 4 and 7% without reducing the Hill coefficients of the Hb D-IHP and Hb A-IHP samples to values below 4.0.

The Interface between Deoxy-Hb D Tetramers—The interface between two Hb D tetramers originally was thought to involve the EF corners from the $\alpha^D$ subunits of the two interacting tetramers. This model is based on three surface residues, Lys$^{\gamma2}$EF1, Gln$^{\gamma78}$EF8, and Glu$^{\gamma82}$F3 which are conserved in the $\alpha^D$ chains but not in the $\alpha^A$ chains (20). Despite the fact that in the absence of IHP, only the Hb D component self-associates (20), the observation of super-cooperativity in both chicken Hbs suggest that the proposed model of the $\alpha^D$-$\alpha^D$ interface may be incorrect. However, the sedimentation experiments of Cobb et al. (20) indicate that the $\alpha$ chains do influence the tetramer-tetramer interaction. The correct model of the tetramer-tetramer interface will require the crystallographic analysis of the T-states of either Hb A or Hb D. Crystals of what should be deoxy-Hb D were obtained that diffract to 6.0-Å resolution when exposed to x-rays produced by a rotating anode generator. Unfortunately, the resulting diffraction pattern could not be indexed due to the presence of crystal plating, and the evidence of 1 unit cell axis which is greater than 300 Å. Nevertheless, a significant amount of information has been inferred from the structural analysis of the R-state of Hb D.

The interface between two deoxy-Hb D tetramers should resemble that observed in the structures of other oligomeric proteins. Analysis of the high-resolution structures of 23 oligomeric proteins shows that a higher percentage of hydrophobic residues are found at the subunit interfaces than are normally located on the surface of the protein (68, 69). Therefore, large, hydrophobic residues (Leu, Ile, Met, Val, Cys, Tyr, and Trp) which are located on the surface of Hb D might contribute to the formation of the tetramer-tetramer interface. Table III lists the 10 hydrophobic residues that have at least 40 Å$^2$ of solvent-exposed surface area. The alignments of the $\alpha$ and $\beta$ chains of Hbs A and D from birds and Hb A from humans revealed that 8 of these residues are retained in many avian Hbs. Three of these residues (Tyr$^{\alpha89}$, Leu$^{\beta10}$, and Met$^{\beta59}$) have small aliphatic or hydrophilic counterparts in human Hb A, suggesting that Tyr$^{\alpha89}$, Leu$^{\beta10}$, or Met$^{\beta59}$ might participate in tetramer-tetramer association. A large, hydrophobic residue (either a phenylalanine or tyrosine) is present at $\alpha89$ in all 20 $\alpha^D$ chains, but is replaced with either a glutamine or histidine in most avian and mammalian $\alpha^A$ chains, respectively. It seems less likely that this residue directly contributes to the association process because both chicken Hbs A and D exhibit super-cooperativity in the presence of IHP. The other two hydrophobic surface residues,
Leu\textsuperscript{A10}(A7) and Met\textsuperscript{E38}(E3), are found in all but three avian Hbs. Leu\textsuperscript{A10} and Met\textsuperscript{E38} are replaced in human Hb by an alanine and lysine, respectively. It is well known that the replacement of a single polar residue by a hydrophobic residue in sickle cell anemia can cause human Hb to polymerize (70). Therefore, it is possible that the tetramer-tetramer interface involves one or more of these hydrophobic surface residues.

An alternative method for locating the tetramer-tetramer interface is to analyze the electrostatic surface potential for chicken deoxy-Hb D. A hypothetical deoxy model was constructed by superimposing each of the αβ dimers onto the corresponding subunits of human deoxy-Hb. The computer program GRASP (51) was used to calculate the surface potentials of the deoxy-Hb D and human deoxy-Hb structures. The most distinctive feature of the surface of chicken Hb D is a large patch of positive charge that corresponds to the IHP-binding site located between the two β subunits (Fig. 6A). A similar positive patch is seen in human Hb (Fig. 6C), but the positive charge is clearly less intense. The increased positive charge allows chicken Hbs to have a greater affinity for organic phosphates than human Hb A as discussed above.

The surface formed by parts of the D and E helices of the β subunits from the chicken Hb D model (residues 45–59) is also significantly different from that of human Hb. This surface in the chicken deoxy-Hb D model appears as an unpolar, oval-shaped protrusion, surrounded by patches of charged residues (Fig. 6D). An apolar patch is also present on the surface of Hb A of the bar-headed goose. Unfortunately, it is not known if bar-headed goose Hb exhibits super-cooperativity in the presence of the IHP-binding site.

The same region in human Hb has a similar shape, but includes charged residues (Fig. 6D). The hydrophobic surface formed by residues 45–59 of the chicken β subunits is the region most likely to participate in contacts between two deoxy-Hb tetramers. An interface involving these residues also makes good chemical sense because this interface would include Leu\textsuperscript{D6}(D6) and Met\textsuperscript{E3}(E3), two hydrophobic residues which would be expected to contribute favorably to the formation of an interface between two Hb D tetramers. Additional contacts may also be involved.

Possible Mechanism for Super-cooperativity—Super-cooperativity in chicken Hbs depends on the formation of a tetramer-tetramer interface which further shifts the conformation to a lower oxygen-affinity state and thus could mediate cooperativity additional to that observed within the α2β2 tetramers. Lampry Hb also exhibits a deoxygenation-linked self-association (71–74) which involves interactions between E helices.\textsuperscript{2} The cooperative mechanism of chicken Hbs could be similar to that observed in Lampry Hb\textsuperscript{2} because the proposed hydrophobic interface is formed by parts of the D and E helices and is located close to the distal histidine. We propose that formation of the tetramer-tetramer interface in chicken Hbs mediates a shift in the E helix of a β subunit in such a way that the distal histidine is pushed further into the heme pocket as it is in lampry deoxy-Hb.\textsuperscript{3} The resulting position of the distal histidine would hinder oxygen binding, thereby reducing the oxygen affinity of this subunit. The binding of oxygen to one of the β subunits would require that the distal histidine move back to an R-state position, thus disrupting the tetramer-tetramer interface and producing α2β2 tetramers of increased oxygen affinity. Experiments are now underway to test this model by crystallizing the deoxy forms of chicken Hbs A and D to define the tetramer-tetramer interface that gives rise to super-cooperativity.

\textsuperscript{2} H. Heaslet and W. E. Royer, private communication.

\textsuperscript{3} H. Heaslet and W. E. Royer, private communication.
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