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# Gel filtration of dilute human embryonic hemoglobins reveals basis for their increased oxygen binding

Lois R. Manning [a], Anthony M. Popowicz [b], Julio C. Padovan [c], Brian T. Chait [c], James M. Manning [a, *]

[a] Department of Biology, Northeastern University, Boston, MA 02115, USA
[b] Information Technology, The Rockefeller University, New York, NY 10065, USA
[c] Laboratory for Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, NY 10065, USA

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ABSTRACT

This report establishes a correlation between two known properties of the human embryonic hemoglobins– their weak subunit assemblies as demonstrated here by gel filtration at very dilute protein concentrations and their high oxygen affinities and reduced cooperativities reported previously by others but without a mechanistic basis. We demonstrate here that their high oxygen affinities are a consequence of their weak assemblies. Weak vs strong hemoglobin tetramers represent a regulatory mechanism to modulate oxygen binding capacity by altering the equilibrium between the various steps in the assembly process that can be described as an inverse allosteric effect.



Adult hemoglobin is the classic example of a protein whose physiological function of $O_2$ transport and its dependence on pH (Bohr Effect) as an example of an allosteric effect can be explained in structural terms as elucidated by the findings of Perutz and his colleagues [1,2] as well as those of many other investigators as described in [3]. Hence, the adult structure is commonly used as the basis to understand the properties of other hemoglobins. For example, the transfer of $O_2$ from the maternal to the fetal circulation is accomplished by two properties of fetal hemoglobin that differ from those of adult hemoglobin, i.e. the single amino acid substitution at the mobile subunit interface involved in the oxy-↔deoxy conformational change [4, 5] and the diminished response of fetal hemoglobin to 2,3-BPG [3] , both of which lead to an increased $O_2$ affinity. Even the abnormal properties of many variant human hemoglobins arising from genetic point mutations can also be understood within the context of the adult hemoglobin structure. However, this model has been less successful in explaining the significantly increased oxygen binding and reduced cooperativities of the embryonic hemoglobins [6–8]. These hemoglobins have many more sequence differences than adult hemoglobins yet all have similar tetrameric structures. Their high $O_2$ affinities, which provide the necessary $O_2$ during the embryonic stage of development when the $O_2$ concentration is extremely low [9], have been attributed to the effects of amino acid substitutions that alter the heme environment to provide increased $O_2$ affinity [7]. However, such conclusions are not supported by convincing data. Likewise, the stepwise nature of the changes in the $O_2$ binding curves of the various embryonic hemoglobins shown below is unexplained. Here we present a novel mechanism addressing these unknowns and based on features in the subunit assembly properties of the embryonic hemoglobins that differ from those of adult hemoglobin as determined by high resolution gel filtration at very dilute concentrations.

It has long been appreciated that the very high $O_2$-affinity of the separate α- and β-subunits (curve to the extreme left in the upper panel of Fig. 1) is dramatically lowered upon their spontaneous assembly to the adult $\alpha_2\,\beta_2$ tetramer (curve to the extreme right) [11, 12]. In addition, the shapes of the curves change from hyperbolic for the individual monomeric subunits indicating the absence of cooperativity to sigmoidal for the tightly assembled adult tetramer arising from its cooperative subunit interactions. These two features– the extent of the shift in position and the shape

* Corresponding author. Department of Biology, Northeastern University, 360 Huntington Avenue Boston, MA 02115 USA.
*E-mail address:* j.manning@neu.edu (J.M. Manning).

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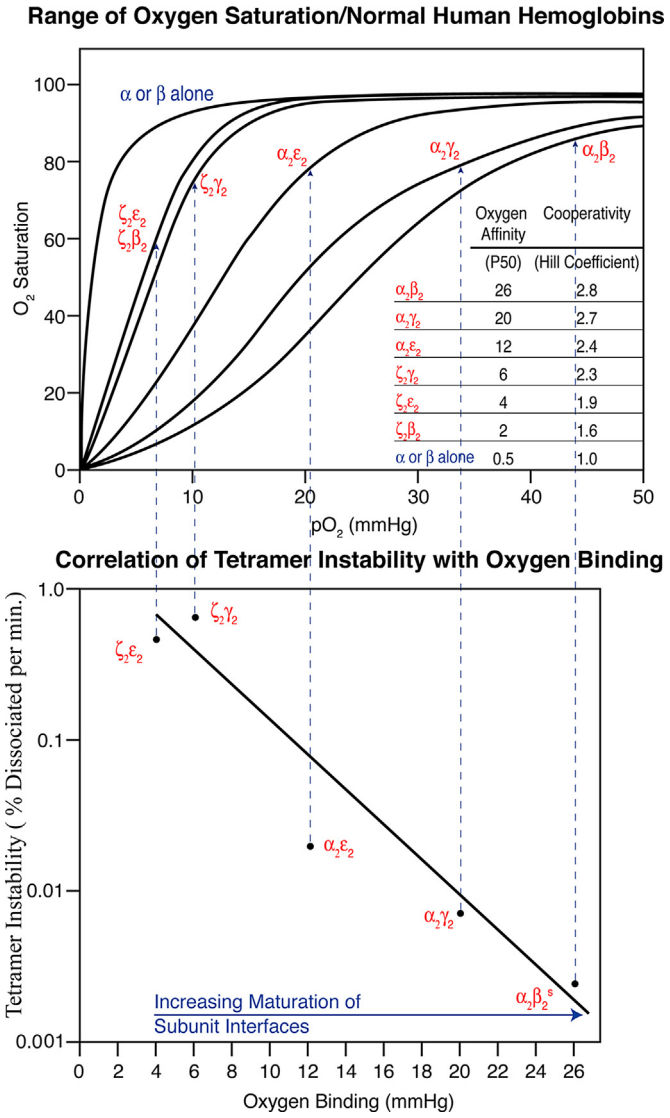


**Fig. 1.** Oxygen Binding Curves of Human Hemoglobins (upper panel) and Correlation of Oxygen Affinities With Tetramer Instability (lower panel).
The oxygen binding curves of all hemoglobins except for $\zeta_2\beta_2$ have been reported in [7] at a concentration of 60 μM tetramer; $\zeta_2\beta_2$ was measured at 7.5 μM [8]. In the bottom panel, the measurements on the y-axis represent the amount of Hb remaining as determined by ion exchange chromatography as described in ref. [10] and expressed as the % Dissociation per min. The values are from Table 2 of ref. [10]: 0.640 ($\zeta_2\gamma_2$), 0.487 ($\zeta_2\varepsilon_2$), average of 0.022 and 0.018 ($\alpha_2\varepsilon_2$), 0.007($\alpha_2\gamma_2$), and 0.0025($\alpha_2\beta_2^s$). The details on how these values were calculated are described in detail in ref. [10]. The values on the x-axis are the P50 values from the inset of Fig. 1, upper panel. The dashed blue vertical lines connecting the upper and lower panels indicate a strong correlation between the affinity of oxygen binding of a given hemoglobin and its rate of dissociation. The solid blue arrow at the bottom of the lower panel shows increasing subunit interface strengths from left to right due to decreasing rates of dissociation.

change in the $O_2$ binding curve– generated by tetramer assembly enable adult hemoglobin to function efficiently as an $O_2$ transporter at atmospheric $O_2$ tension. The reason for the less extreme shift in the $O_2$ binding curve of fetal hemoglobin is described above [4, 5]. But why do the embryonic hemoglobins have significantly increased $O_2$ binding compared to that of adult Hb (Fig. 1, upper panel) thus enabling them to function at the very low $O_2$ tension present during the early stages of development [8, 9]? We approached this question by comparing what is known about the assembly process of the adult Hb tetramer [1, 2] and determining how the embryonic hemoglobins differ from this model. High resolution gel filtration at very dilute concentrations was instrumental in resolving this question.

For adult Hb, tetramer assembly is initiated when α and β monomers **(M)** are bound together by a very strong interface to form dimers (**D**), two of which then join together with a mobile interface facilitating the transition between the two tetramer conformations ($\mathbf{T^{deoxy}}$ and $\mathbf{T^{oxy}}$) described in the assembly equation below. (Here **T** does not refer to the "tense" state, i.e. we are not using the R/T terminology). The ratio of $\mathbf{T^{deoxy}/T^{oxy}}$ is defined as **L**, the allosteric constant [1, 2], which is an intrinsic property of each hemoglobin type. Although the value of **L** for the embryonic hemoglobins is lower than that for adult Hb [7], no mechanism is known; below we present one. Whereas the equilibria between tetramers and dimers **(T/D)** and between the two $\mathbf{T^{deoxy}/T^{oxy}}$ conformational states in the equation below are completely reversible for all human hemoglobins [13–15], the equilibrium between monomers and dimers **(D/M)** for adult [16] and fetal hemoglobins is practically irreversible. Hence, after dimers are formed, monomers play no further functional role for these two hemoglobins.

$$4\,M \leftrightarrows 2\,D \leftrightarrows T^{\,oxy} \leftrightarrows T^{\,deoxy}$$

During the $\mathbf{T^{deoxy}/T^{oxy}}$ conformational switch for these two hemoglobins, the **T/D** interfaces change interactions between amino acid side chains but the **D/M** interface does not rearrange [1–2]. In the equation above, energetically more favorable states increase and $O_2$ binding decreases from left to right so that $\mathbf{T^{deoxy}}$ has the strongest subunit interactions and has no bound $O_2$. Although this information has been known for many years for adult Hb [1,2], there is less information on embryonic hemoglobins.

The description for the individual steps for adult Hb in the equation above has been tacitly assumed to apply to all hemoglobins. We reported earlier [4, 10] that the **T/D** equilibria for the fetal and embryonic hemoglobins differ significantly from that of adult hemoglobin [13–15] and each equilibrium was found to be completely reversible. Of particular interest, however, was the finding that the embryonic hemoglobins deviate from the adult hemoglobin paradigm regarding their **(D/M)** interfaces, which are relatively weak in comparison to those of adult and fetal hemoglobins [10, 17–19] as shown in Fig. 2. At the high protein concentrations, i.e. micromolar, usually employed for gel filtration, adult hemoglobin shows a single symmetrical peak in the tetramer position whereas the embryonic hemoglobins exhibit a broader tetrameric component with a trailing species between the dimer and monomer positions [10]. These slight differences would not usually prompt further study but under closer examination at nanomolar concentrations, this behavior is accentuated as shown in Fig. 2. Adult and fetal hemoglobins (upper two panels) do not dissociate beyond the dimer stage but each of the embryonic hemoglobins (bottom two panels) does so. However, the similar dissociation profiles for embryonic $\zeta_2\gamma_2$ and $\alpha_2\varepsilon_2$ (blue lines in lower two panels of Fig. 2) are not what one would expect from their very different dissociation rates shown in Fig. 1. This apparent discrepancy is very likely due to an unusual property of $\zeta_2\gamma_2$ not found for the other hemoglobins, i.e. the monomeric γ subunit formed by dissociation of the **D/M** interface undergoes self assembly to $\gamma_2$ dimers and $\gamma_4$ tetramers as reported by others [20, 21], thus shifting the gel filtration profile to the left.

Weak **D/M** interfaces will shift the above equation for the embryonic hemoglobins to the left resulting in increased $O_2$ binding. This behavior can be described as an *inverse allosteric effect* since it acts in a manner opposite that of usual allosteric regulators such as BPG and protons, which are exogenous regulators that *first* bind to $\mathbf{T^{deoxy}}$ hemoglobin to shift the above equation to the right *and then*

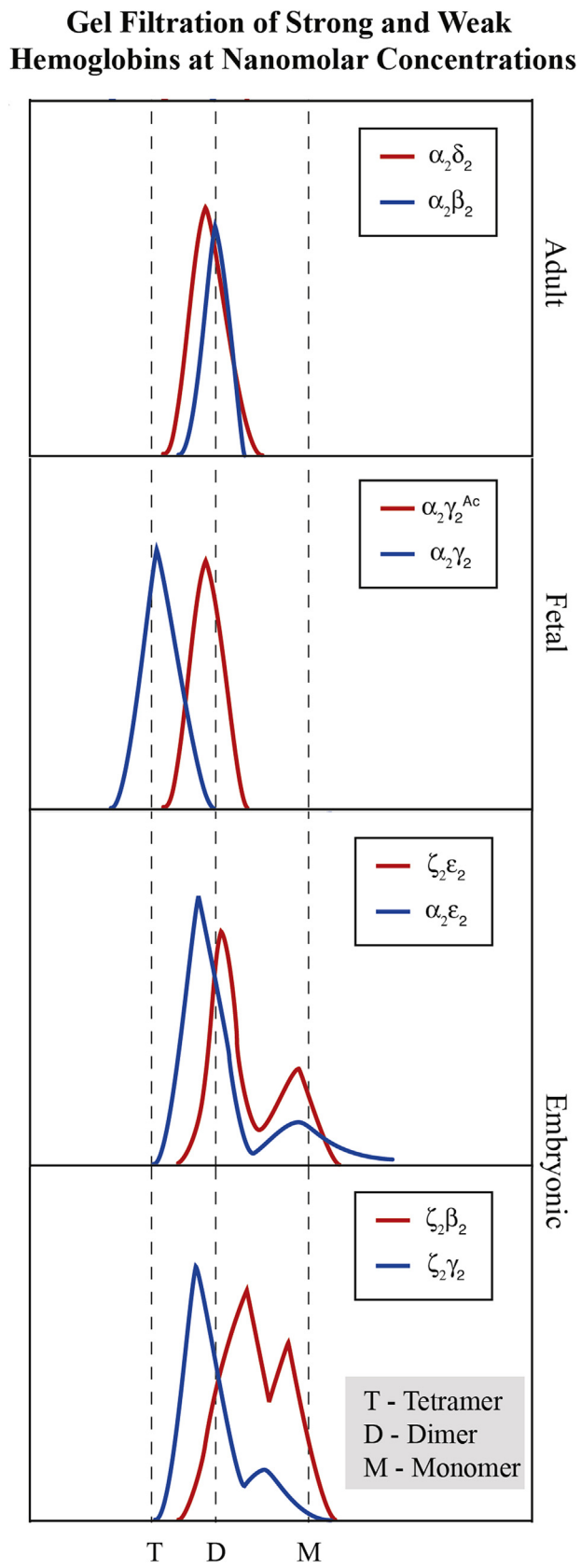


**Fig. 2.** Gel Filtration of Human Hemoglobins at Nanomolar Concentrations.
Concentrations of CO-hemoglobin in the range of 10–100 nM were applied to Superose-12 on a Pharmacia FPLC system as described previously [18]. Fetal. Hb F, which has a very low **T/D** Kd, remains tetrameric but natural acetylated HbF1 and adult Hb A, which have much higher **T/D** Kd values are mainly dimeric [22]. The embryonic hemoglobins show significant monomer species. The dissociation profile of embryonic $\zeta_2\gamma_2$ shown in the lower panel is very likely skewed to the left towards higher molecular weight components due to the formation of $\gamma_2$ dimers and $\gamma_4$ tetramers formed by re-assembly of $\gamma$ monomers as explained in the text. The strong $\gamma_2$ dimers are not in a facile equilibrium with $\gamma$ monomers but are with $\gamma_4$ tetramers as shown by two different laboratories [20, 21]. Indeed, $\gamma_4$ (known as Hb Barts) inadvertently crystallized during efforts to crystallize $\zeta_2\gamma_2$.[21].

result in release of additional $O_2$. In an *inverse allosteric effect* the opposite sequence occurs– the embryonic hemoglobin assemblies first undergo redistribution (due to their intrinsically weak intersubunit contacts at the **D/M** interface not requiring an exogenous regulator) causing a shift of the above equation to the left. Thus, the $\mathbf{T^{deoxy}}$ structure would be weakened favoring the $\mathbf{T^{oxy}}$ structure resulting in an increased $O_2$ affinity for the embryonic hemoglobins. This mechanism differs from that involved in the increased $O_2$ affinity of fetal Hb F (see Fig. 1, upper panel), which also has a favored $\mathbf{T^{oxy}}$ structure but one that results from its stronger **T/D** interface compared to that of adult Hb A [4, 5]. Additional such examples may be found in other protein assemblies by using the technique of high resolution gel filtration at dilute protein concentration.

The major factor contributing to the stepwise nature in the shifts of the $O_2$ binding curves of the embryonic hemoglobins shown in Fig. 1, upper panel, is the variable binding affinities of the ζ- and α-monomeric subunits when each pairs with either ε-, γ-, δ-, or β-monomeric subunits to form dimers, e.g., αβ is the strongest dimer but ζβ is the weakest; all other subunit combinations are intermediate between these two, spanning a range of several hundred fold [10]. This variable efficiency with which subunits combine to form dimers could be relevant in the developmental progression from embryonic to fetal and then to adult hemoglobins due to increasing maturation of their interfaces.

The assemblies involving the embryonic hemoglobin are further weakened by the presence of an acetyl group on the N-terminus of the ζ-subunit analogous to that found for the minor fetal hemoglobin component acetylated on its γ-subunit N-terminus [20, 21]. In general, the acetyl group weakens subunit assembly by 20–30 fold [22, 23]. This effect is readily apparent in the second panel of Fig. 2 where at very dilute concentrations, $\alpha_2\gamma_2$ remains tetrameric but $\alpha_2\gamma_2^{Ac}$ is dimeric. Had the usual high protein concentration been used, this difference would not have been observed. Thus, the embryonic pairs are energetically weaker than the fetal or adult pairs accounting for their lower value of **L** [7]. As a consequence, their $O_2$ binding curves also shift in the same direction to intermediate positions between that of the tightly assembled low affinity $\boldsymbol{\alpha_2\beta_2}$ adult tetramer (curve to the far right) and that of the high affinity monomers (curve to the far left). Thus, the positions of the $O_2$ binding curves for the embryonic hemoglobins are a result of their inefficient tetramer assembly due to the changes of the **D/M** equilibria leading to a left shift in the above equation. A weaker assembly structure renders it prone to dissociation upon gel filtration. However, a significant presence of monomers at the prevailing hemoglobin concentration in the embryonic red cell seems unlikely.

As shown by the vertical dashed blue arrows connecting the lower panel to the upper panel of Fig. 1, there is a good correlation between the rate of dissociation (taken from Table 2 of ref 10) essentially representing the **D/M** equilibrium and oxygen binding for each hemoglobin (expressed as $P_{50}$ values). This correlation represented by this vertical blue line has not previously been made and demonstrates the reason for the stepwise nature of the shifts in the $O_2$ binding curves shown in the upper panel of Fig. 1.

Cooperativity, which arises during the transition between the two $\mathbf{T^{deoxy}}$ **and** $\mathbf{T^{oxy}}$ states, decreases for the embryonic

hemoglobins (stepwise lowered Hill coefficients shown in Fig. 1) because the concentrations of the two T states change when the equation above shifts to the left generating a less sigmoidal binding curve. The progressive nature of the cooperativity changes is a result of the stepwise shift from non-cooperativity of the single subunits to full cooperativity of adult tetrameric Hb.

Hb $\alpha_2\varepsilon_2$, which is comprised of adult and embryonic subunits, represents a transitional Hb with intermediate values of oxygen affinity ($P_{50}$), and cooperativity (Hill coefficient) shown in the upper panel, which correlate with its tetramer instability shown in the lower panel. The horizontal solid blue arrow at the bottom of the lower panel indicates the increasing subunit interface strengths and decreasing free energies of the hemoglobins present during the embryonic, fetal, and adult stages of development respectively, representing the progression from less mature to more mature tetramers [10].

Myoglobin, another member of the human globin family, is a monomer with an extremely high $O_2$ affinity like that of the individual hemoglobin subunits [10, 17–19]. However, it does not participate in subunit assembly thus retaining a very high $O_2$ binding capacity required of metabolically active muscle tissue. Hence, myoglobin and the individual subunits represent the completely dissociated state consistent with their very high oxygen affinities.

The results described here demonstrate that the variable $O_2$ binding shown in the upper panel of Fig. 1 comprise a spectrum that depends on whether the subunits assemble tightly (adult and fetal hemoglobins), weakly (embryonic hemoglobins), or not at all (myoglobin). These properties are readily observed when gel filtration is performed at very dilute protein concentrations, which provides a more comprehensive view of the assembly process. Hence, the assembly equation above represents a gradient of high (monomeric subunits) to low (tightly assembled adult tetramers) $O_2$ binding strengths. These results also suggest the possibility that external agents that can specifically perturb the **D/M** subunit interface interactions may be a means to alter $O_2$ binding in situations where this would be beneficial. The theme correlating protein assembly strength to modulation of its function may also apply to other protein systems.

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## Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

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