FOR THE RECORD

Developmental expression of human hemoglobins mediated by maturation of their subunit interfaces

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Abstract: Different types of human hemoglobins (Hbs) consisting of various combinations of the embryonic, fetal, and adult Hb subunits are present at certain times during development representing a major paradigm of developmental biology that is still not understood and one which we address here. We show that the subunit interfaces of these Hbs have increasing bonding strengths as demonstrated by their distinct distribution of tetramers, dimers, and monomers during gel filtration at very low-Hb concentration. This maturation is mediated by competition between subunits for more favorable partners with stronger subunit interactions. Thus, the protein products of gene expression can themselves have a role in the developmental process due to their intrinsic properties.

Keywords: hemoglobin; subunit interfaces; globin gene clusters; development

Introduction

Hemoglobin (Hb) is one of the best-known proteins regarding its structure/function relationship for binding and delivering O_2.[1,2] The adult type is most often studied; results on the embryonic and fetal types are usually interpreted within its framework. The expression of the eight different types of normal human Hbs during the embryonic, fetal, and adult stages of life (Fig. 1) represents a major model of developmental biology, which is currently explained by the "switching" on and off of the various globin genes, known as ontogeny.[4] The process is initiated by various transcription factors that interact with upstream regulatory regions of the two globin gene clusters[3] (Fig. 1). These regions, which are shown in gray boxes, are referred to as HS-40 for the α-like globin genes and β-LCR for the β-like globin genes. They are typified by their sensitivity to digestion by DNAase so they are considered to be open areas of chromatin accessible to regulatory factors that

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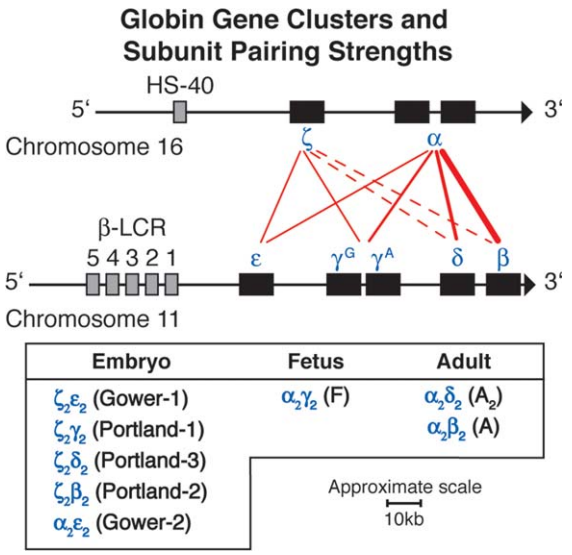

Figure 1. Globin gene clusters. The α-like globin genes are located on chromosome 16 and the β-like globin genes are located on chromosome 11. The gray boxes labeled HS-40 on chromosome 16 and β-LCR (5,4,3,2,1) on chromosome 11 are regions that are sensitive to digestion by nucleases enabling the transcription of the individual globin genes (black boxes) in the order 5′ → 3′ to produce the eight globin subunits (blue Greek letters), whose common names and the stages of development at which they are present are shown in the inset (adapted from Higgs *et al*.[3]; permission to use Figure 1 of Ref. 3 obtained from Wiley-Blackwell.). The red lines between the globin subunits represent subunit interactions in each dimer and are from our data in Figure 2, which has been superimposed on a modified Figure 1 of Higgs *et al*.[3] The widths of these red lines and whether they are continuous or dashed indicate the strengths of the subunit interactions of a particular dimer pair.

control transcription of the downstream globin genes (black boxes in Fig. 1). This model for initiation of gene expression is supported by a significant body of data.[5] On the other hand, termination of gene expression exemplified by the loss of certain Hb types is less well understood although the relative stabilities of embryonic, fetal, and adult mRNA are considered to be involved in this process.[6]

In the model described above, no attention is given to the Hbs themselves expressed during these stages as having any regulatory role. Although it is well known that these Hbs have different affinities for oxygen,[7,8] which is crucial during the various stages of prenatal and postnatal life, there have been fewer studies on their subunit–subunit interactions. In fact, the prevailing view is that all normal human Hbs are structurally alike and there is little consideration of strong or weak subunit interactions as being of importance.[7,8] However, using direct and very sensitive techniques,[9,10] we found large differences among them regarding the structural strengths at their subunit interfaces as shown below by their strikingly different patterns during molecular sieve chromatography (gel filtration) when performed at concentrations that do not obscure these intrinsic differences, that is, much lower than their equilibrium constants. These gel filtration patterns represent distinct phenotypes for the embryonic, fetal, and adult Hbs that appear to be linked to the developmental process. The use of the term phenotypes to describe subunit assembly has been used previously[11] but in a different context.

The results in this communication follow directly from our earlier reports on the tetramer–dimer and dimer–monomer subunit interfaces of these Hbs where the individual constants for the dissociation of these interfaces were determined.[12,13] In this article, these equilibria have been studied together in a single measurement as linked events. Information on individual equilibria enabled us to choose a Hb concentration where differences in their subunit interface bonding strengths would be manifested; if too high a Hb concentration had been studied, the equilibria of all the Hbs would have been shifted toward the tetrameric state thus obscuring any differences between them.

Results and Discussion

The molecular weights of the human embryonic, fetal, and adult Hb tetramers are within a few hundred mass units of 64,000. The subunit compositions and their common names are shown in Figure 1. Each Hb migrates in the same position as a tetrameric species during gel filtration when the concentrations are in the millimolar range, which exceeds their subunit dissociation constants. However, we found that if the concentrations are reduced to the nanomolar range, which is below their tetramer dissociation constants, the elution patterns are radically different from one another (Fig. 2) indicative of significant variability in their subunit interface strengths as shown by the red lines that have been added to the scheme of Higgs *et al*.[3] shown in Figure 1.

Gel filtration patterns of nanomolar concentrations of hemoglobins

The high-resolving power of the Superose gel filtration matrix and the absence of excessive peak broadening account for the high degree of precision and reproducibility of the elution patterns are shown in Figure 2 (0.3% as described in Methods and Materials section). The embryonic Hbs in Figure 2(A,B) show a significant percentage of dimers and monomers. In any given panel of Figure 2, the Hb shown in red is more dissociated than the Hb in blue. The most dissociated of all the normal Hbs $\zeta_2\beta_2$ (Hb Portland-2) is not found normally but present only in the complete absence of α-subunits (as in α-thalassemia major). Thus, α-subunits have a higher affinity for β-like subunits than do ζ-subunits. Embryonic Hb $\zeta_2\delta_2$ (Hb Portland-3) has only been reported as a

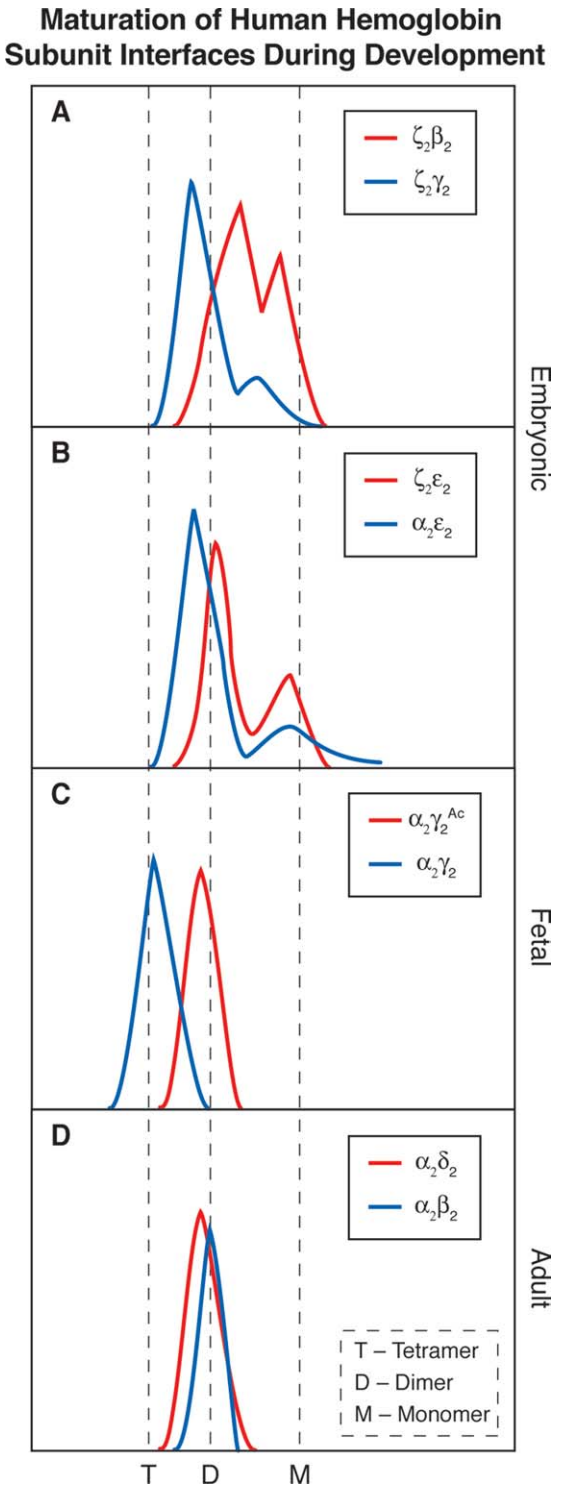

Figure 2. Gel filtration patterns of hemoglobins at nanomolar concentrations. The experimental conditions are given in the text. In any given panel, the more dissociated hemoglobin is shown in red.

band on a gel in α-thalassemia major[14] so it is also considered as a very highly dissociated Hb. It is likely that these two Hbs are not found normally because their subunit interfaces are so weak (Fig. 1, dashed red lines) that other Hbs with stronger subunit interfaces are favored (Fig. 1, solid red lines) as described next. In general, the gel filtration profiles in Figure 2 become progressively less complex starting from the embryonic Hbs [Fig. 2(A,B)] through the fetal Hbs [Fig. 2(C)] and finally for the adult Hbs [Fig. 2(D)].

Role of subunit interfaces during development

The very weak subunit interactions of the embryonic Hbs shown in Figure 2(A,B) are consistent with their short half-lives *in vivo* as monomeric subunits are unstable.[15] In contrast, the fetal and adult Hbs shown in Figure 2(C,D), respectively, do not dissociate to monomeric subunits to any measurable degree and hence have longer durations. This is clear evidence that Hbs containing α-subunits have much stronger interactions with their partner subunits than do those containing ζ-subunits. Fetal Hb (HbF, $\alpha_2\gamma_2$) is the least dissociated, that is, most associated of all the human Hb tetramers [Fig. 2(C), blue curve] consistent with its rapid onset at 6 weeks[16,17] due to its very low-free energy as calculated from its dissociation constant.[13] Subsequently, slow post-translational acetylation at the N-terminus of its γ-subunit (HbF$_1$, $\alpha_2\gamma_2^{Ac}$) weakens its very strong subunit interactions [Fig. 2(C), red curve][18] consistent with the gradual demise in HbF production and its replacement by HbA around the time of birth. In addition to this attenuating effect of acetylation, there are polymorphisms at positions 75 and 136 of the two γ-genes,[19] making HbF a multicomponent system in contrast to HbA, which is a single component [Fig. 2(D), blue curve] without modifications or polymorphisms. In addition, the γ-subunits of HbF undergo unproductive γ_2-homodimer formation, which limits the amount of functional αγ heterodimers.[20] The other adult Hb (HbA$_2$, $\alpha_2\delta_2$) displays an abnormal equilibrium between dimers and monomers as shown by its wide peak [Fig. 2(D), red line] and also by the displacement of its peak width maximum from its tetramer–dimer K_d value.[21] In contrast, HbA formation has a much narrower peak width [Fig. 2(D), blue line] whose maximum coincides with its tetramer–dimer K_d value[21] making it the most favorable Hb species.

Competition among subunits

As the globin genes on chromosomes 16 and 11 are transcribed in the 5′ → 3′ direction (Fig. 1), the ζε and ζγ embryonic globin subunit pairs are formed at the earliest times and at low-initial concentrations. The results in Figure 2(A,B) show that these Hbs have very weak subunit interactions, which are depicted as thin red lines between the subunit partners in Figure 1. The fetal and adult Hbs formed next have stronger subunit interactions [Fig. 2(C,D)], which are represented by broader red lines between the αγ, αδ, and αβ subunits in Figure 1. The overall pattern of the lines connecting the subunits

in Figure 1 shows a reciprocal relationship, that is, subunit interaction strength for Hb pairs containing ζ-subunits decreases 5′ → 3′, whereas for those subunit pairs containing α-subunits, the bonding strength increases 5′ → 3′, for example, ζβ is the weakest dimer and αβ is the strongest dimer, representing a progressive directionality in the subunit pairing pathway.

These results provide an explanation for "gene switching" in Hb ontogeny based on the properties of the globin subunits themselves and can be considered as complementary with the current models. In the first "switch," the strong αγ pair is formed initially and prevails over the weaker αε, ζε, and ζγ pairs. Slow post-translational acetylation of γ-subunits leads to weaker $\alpha\gamma^{Ac}$ pairs[18] resulting in the second "switch" when β-subunits are expressed to form the αβ pair. The preferential assembly of $\alpha_2\beta_2$ over $\alpha_2\gamma_2$ has been demonstrated by *in vitro* studies involving mixing of isolated Hbs.[12,13,22] Thus, when the weak embryonic Hbs $\zeta_2\beta_2$, $\zeta_2\gamma_2$, and $\alpha_2\varepsilon_2$ are mixed, they slowly dissociate and reassemble to form $\alpha_2\beta_2$ (HbA), the strongest tetramer, through subunit exchange, that is,

$$\zeta\boldsymbol{\beta} + \zeta\gamma + \boldsymbol{\alpha}\varepsilon \rightarrow \boldsymbol{\alpha\beta}$$

Neither $\alpha_2\gamma_2$ nor $\zeta_2\varepsilon_2$, the other possible tetramers, are formed. These results are in accordance with the directionality indicated by the widths of the red lines in Figure 1, which represent the subunit interaction strengths of the dimers. The same conclusion regarding the preferential assembly of $\alpha_2\beta_2$ over $\alpha_2\gamma_2$ has been reached by *in vivo* studies using a yeast expression system in which α, β, and γ genes in different orders were allowed to compete; HbA ($\alpha_2\beta_2$) was consistently formed in greater amounts than ($\alpha_2\gamma_2$) HbF, regardless of gene order.[13] Hence, a major driving force in the developmental formation of Hbs may be competition among subunits to form more favorable pairs (and eventually tetramers) with progressively stronger subunit interfaces. However, even though we have demonstrated subunit competition/exchange, we have no evidence that such a mechanism is physiologically operative. The pairings of the globin subunits shown by the variable widths of the red lines in Figure 1 indicate that the globin subunits that are not passive structures but actively seek optimal partners to dynamically drive the developmental process. It is likely that this theme is repeated often in other protein assemblies that involve subunit interfaces of variable strengths.

Physiological consequences of variations in subunit assembly

It is important to emphasize that even though the differences in subunit interactions within the Hb family shown in Figure 2 are not readily discernible at high-Hb concentrations, they are nonetheless always present; comparison of the ease of formation of $\zeta_2\beta_2$ and $\alpha_2\gamma_2$ tetramers, the weakest and strongest of all the normal human Hbs, respectively, shows that $\zeta_2\beta_2$ becomes tetrameric much less readily requiring a significantly higher concentration than does $\alpha_2\gamma_2$.[12]

A novel finding of this communication is that whereas fetal and adult Hb tetramers are made up of strong dimers [Fig. 2(C,D)], embryonic Hb tetramers consist of weak dimers that dissociate further to monomers [Fig. 2(A,B)] destabilizing the functional tetrameric state. This fundamental difference affects their physiological properties resulting in increased O_2 binding, reduced cooperativity, and decreased response to allosteric regulators such as 2,3-DPG, all of which are known differences among the embryonic, fetal, and adult Hbs.[8] Such variations, which provide the essential O_2 environment during the various stages of development, are completely consistent with the findings described here.

Methods and Materials

Hemoglobin purification and characterization

HbF (HbF_o) and its acetylated counterpart (HbF_1) were purified from human umbilical cord blood; adult HbA and adult HbA_2 were purified from peripheral blood drawn by venepuncture from normal individuals.[9,10] The embryonic human Hbs were purified from the blood of transgenic mice[12,13] that had been transfected with the human embryonic globin genes.[23] All Hbs were homogenous as determined by high-resolution isoelectric focusing and analytical FPLC; each had the correct mass as measured by mass spectrometry.

High-resolution gel filtration

The Hb samples were either in the CO-liganded or the O_2-liganded state in a sample volume of 100 μL applied to a Superose-12 HR 10–30 column on a Pharmacia FPLC system.[9,10] The applied Hb concentrations were in the range of 10–100 n*M*, which is near or below the tetramer–dimer dissociation constants for all the Hbs shown in Figure 2. Hence, the tetrameric state would not predominate thus favoring the possibility of detecting dimer dissociation to monomers especially for weak dimers. The elution buffer was 150 m*M* Tris-Ac, pH 7.5, at a flow rate of 0.4 mL/min. Samples were diluted about sixfold during the elution. The detection system was a Pharmacia online mercury lamp set at 405 n*M* with a 5 mm flow cell. As the spectrum of the eluted Hb was identical to that of the applied Hb, there was no dissociation of the heme from the globin nor was there any oxidation of the iron to met Hb. Replicate analysis showed that the variation in the peak position was within 0.3%.[24] The eluted peaks were subjected to

complete mathematical analysis. The standards used to determine the positions of the tetramer, dimer, and monomer in Figure 2 were Hb crosslinked between the N-terminal residues of the α-subunits by a diisothiocyanatobenzene sulfonic acid,[9,10] dimeric Hb Rothschild,[25] and the hydroxymercuribenzoate derivative of the isolated α-subunits, respectively.[15,26]

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