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New Concept

Energetic Differences at the Subunit Interfaces of Normal Human Hemoglobins Correlate with Their Developmental Profile[†]

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ABSTRACT: A previously unrecognized function of normal human hemoglobins occurring during protein assembly is described, i.e. self-regulation of subunit pairings and their durations arising from the variable strengths of their subunit interactions. Although many mutant human hemoglobins are known to have altered subunit interface strengths, those of the normal embryonic, fetal, and adult human hemoglobins have not been considered to differ significantly. However, in a comprehensive study of both types of subunit interfaces of seven of the eight normal oxy human hemoglobins, we found that the strengths, i.e., the free energies of the tetramer−dimer interfaces, contrary to previous reports, differ by 3 orders of magnitude and display an undulating profile similar to the transitions ("switches") of various globin subunit types over time. The dimer interface strengths are also variable and correlate linearly with their developmental profile. Embryonic hemoglobins are the weakest; fetal hemoglobin is of intermediate strength, and adult hemoglobins are the strongest. The pattern also correlates generally with their different O_2 affinities and responses to allosteric regulatory molecules. Acetylation of fetal hemoglobin weakens its unusually strong subunit interactions and occurs progressively as its level of expression diminishes and adult hemoglobin A formation begins; a causal relationship is suggested. The relative contributions of globin gene order and competition among subunits due to differences in their interface strengths were found to be complementary and establish a connection among genetics, thermodynamics, and development.

Protein subunit assemblies range from small complexes such as hemoglobin (Hb)[1] tetramers to large networks such as those in cell signaling pathways. Protein assemblies are held together in large part by protein−protein interactions involving subunit interfaces, which have bonding strengths that define their affinity for partner subunits, the duration of this interaction, and whether certain subunits can exchange with other assemblies to generate enhanced or even different functions. It is these topics that we describe in this article using normal human hemoglobins as an example; in such a small complex, general principles of subunit assembly might be readily observed. In a protein network, both the strength and duration of the initial interaction must be sufficient to enable reinforcing interactions to occur. Mutant subunits can have deleterious effects on protein assemblies either by creating aberrant subunit interfaces such as those associated with abnormal hemoglobins (*1*−*3*) or by enhancing or decreasing protein subunit interactions in cell signaling pathways as in some human carcinomas thereby altering their normal duration and progression (*4*).

Hemoglobin Ontogeny. Human hemoglobins comprised of ζ- or α-subunits paired with ε-, γ-, δ-, or β-subunits representing embryonic, fetal, and adult hemoglobins have nearly identical overall structural architecture in their tetrameric states (Figure 1A). The individual hemoglobin subunits are temporally expressed during normal development (Figure 1B) in a pattern that reflects their gene order, which exists in two separate gene clusters termed α-like and β-like on human chromosomes 16 and 11, respectively (Figure 1C) (*5*−*7*). This transcription process is preceded by relaxation of the chromatin structure where the globin genes reside to permit access by transcription factors. These early events are considered independent of the late process of protein subunit assembly whose intersubunit energetics we find to differ among the normal human hemoglobins in a pattern that reflects their temporal expression, thus linking hemoglobin structure (Figure 1A) and its developmental profile (Figure 1B).

There are eight possible combinations of globin subunits that are formed first as dimer pairs and then as tetramers; their names are given in Figure 1D. The transition among hemoglobin types over time (Figure 1B) represents a major paradigm for developmental biology, although some of its aspects such as the transition ("switch") from ζ- to α-subunit expression and from ε- to γ-subunit expression during the embryonic stage and that from γ- to β-subunit expression during fetal life are poorly understood (*8*). Although differences have been reported in bonding strengths involving subunit interfaces of many mutant hemoglobins (*2*), normal human hemoglobins have not been considered to vary significantly in their interface properties (*9*). Therefore, no consideration has been given to post-translational contributions in the developmental process that are distinct from

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[1]Abbreviations: Hb, hemoglobin; DPG, 2,3-diphosphoglycerate; Ac, acetyl; LCR, locus control region; URE, upstream region element.

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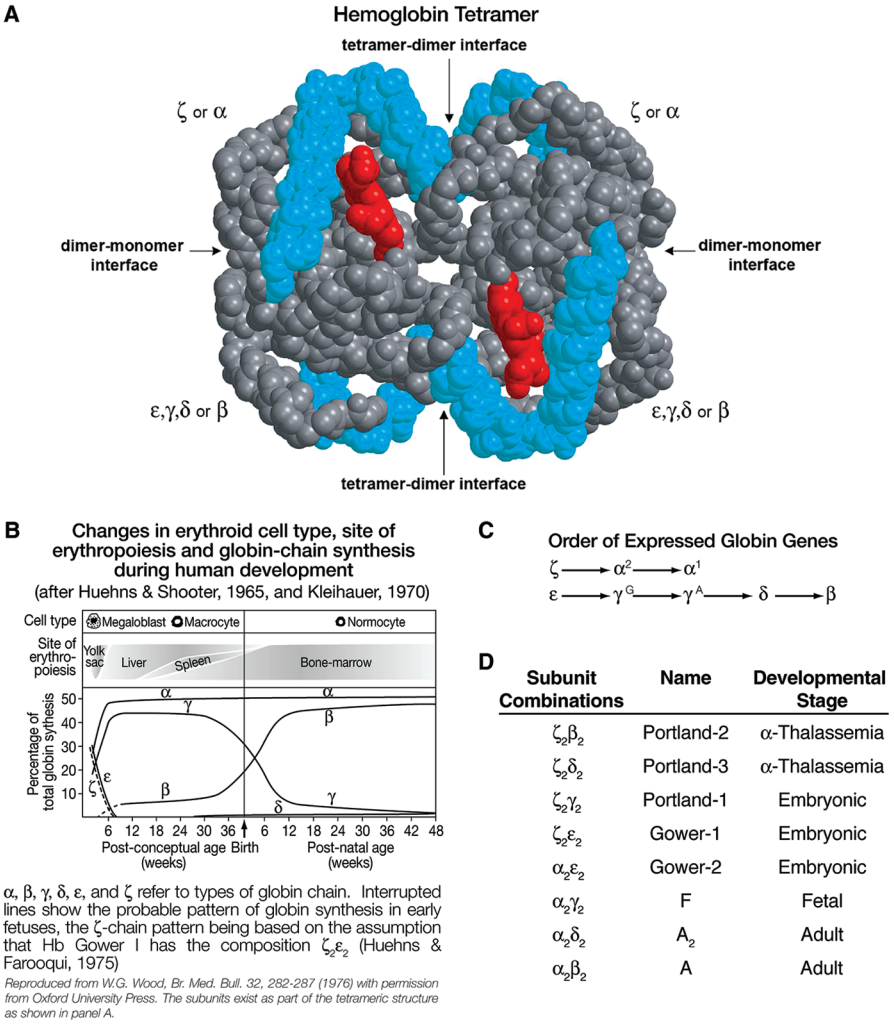

Subunit Combinations	Name	Developmental Stage
$\zeta_2\beta_2$	Portland-2	α-Thalassemia
$\zeta_2\delta_2$	Portland-3	α-Thalassemia
$\zeta_2\gamma_2$	Portland-1	Embryonic
$\zeta_2\varepsilon_2$	Gower-1	Embryonic
$\alpha_2\varepsilon_2$	Gower-2	Embryonic
$\alpha_2\gamma_2$	F	Fetal
$\alpha_2\delta_2$	A_2	Adult
$\alpha_2\beta_2$	A	Adult

FIGURE 1: (A) Hemoglobin tetramer with the location of the tetramer−dimer and dimer−monomer interfaces indicated by arrows. The cyan color indicates the E and F helices, whereas the rest of each subunit is colored gray. The heme is colored red. The top subunits can be either ζ or α and the bottom subunits ε, γ, δ, or β. Courtesy of W. Royer, Jr. (B) Developmental changes in the expression of Hb types as a function of time. The individual subunits are shown, although they are actually present as tetramers with partner subunits. (C) Order of globin subunit expression. Each gene is separated by 10−20 kb of untranscribed DNA. (D) Nomenclature for eight normal human hemoglobins. Those designated as occurring in the α-Thalassemia syndrome are found only in the total absence of α-subunit expression ("hydrops fetalis").

transcriptional events. Most studies of human hemoglobins have involved the adult type, with many fewer reports on the embryonic or fetal hemoglobins in part because of a lack of availability, especially of the embryonic types. This predominance of adult hemoglobin data has thus engendered the notion that all normal human hemoglobins have similar subunit interface association and dissociation properties since their overall structural architectures are so similar. However, there are no careful measurements to support this view, and in this work, we show that this widely held belief is erroneous. On the other hand, oxygen affinity differences and responses to allosteric regulation for the various normal hemoglobins, which are conferred by tertiary and quaternary structural changes (*10*), are well-known to be critical for proper development. However, these physiological properties are unlikely to be the reason for the temporal appearance of the various hemoglobins during development.

Subunit Interfaces in Normal Human Hemoglobins. The location and bonding contacts of the two major types of subunit interfaces in the oxy and deoxy conformational states of HbA are well-known (Figure 1A) (*10*, *11*). The tetramer−dimer interface, which is flexible and rearranges its subunit contacts during the oxy−deoxy conformational switch, has been studied extensively (*10*−*14*) and can be considered as the modulator of O_2 binding and delivery. The dimer−monomer interface, which is very rigid and does not move significantly during the conformational switch (*10*, *11*), mediates subunit recognition when the various subunits compete for partners.

Before the bonding strengths of both types of interfaces for the normal human hemoglobins (in the liganded state) were measured, it was first necessary to obtain the purified and well-characterized proteins. Human HbA and fetal HbF are readily purified from adult blood and umbilical cord blood, respectively, but the embryonic hemoglobins are not easily obtained. He and Russell (*15*) circumvented this problem by using blood from transgenic mice transfected with the human hemoglobin embryonic genes. It was thus possible for us to study the family of normal human hemoglobins simultaneously and comprehensively using highly precise methodology and complete data analysis.

Tetramer−Dimer Interfaces of Normal Hemoglobins Have Variable Strengths. In contrast to other reports (*9*), we find that the tetramer−dimer dissociation constants of seven of the eight normal hemoglobins vary by up to 3 orders of magnitude (*16*). The free energy values of this interface for these hemoglobins as well as that of the acetylated form of HbF

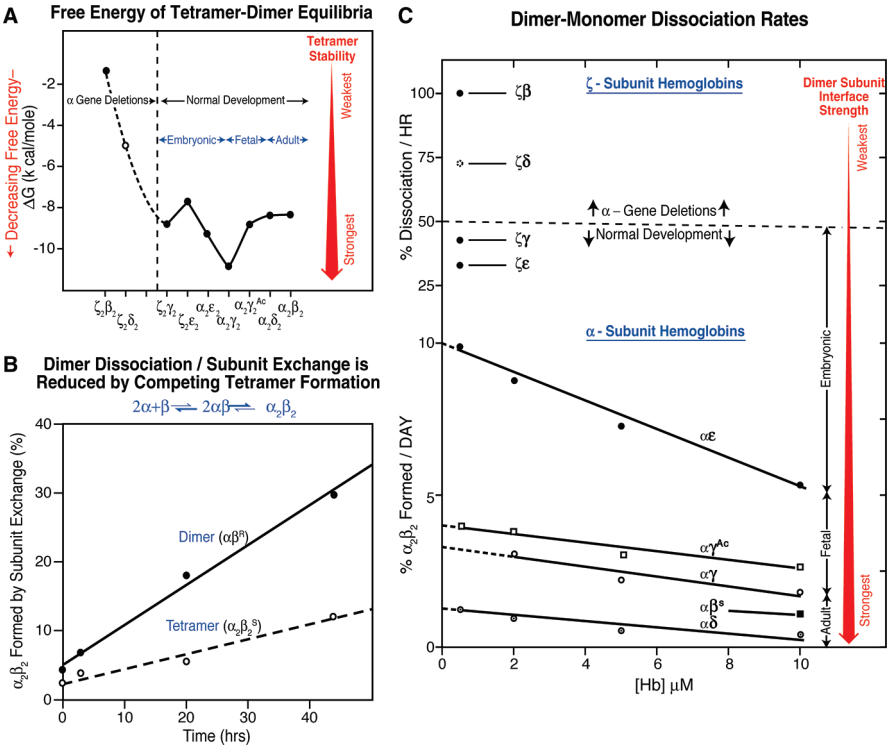

FIGURE 2: (A) Free energy values for the tetramer−dimer equilibria for the normal human hemoglobins calculated from the dissociation constants at pH 7.5 (*16*). Hb Portland-3 ($\zeta_2\delta_2$) is unavailable, so its value is approximate and designated with an empty circle. HbF_1 is shown as $\alpha_2\gamma_2^{Ac}$. (B) Effect of the tetrameric state on the exchange of monomeric subunits from the dissociated dimeric state of Hb Rothschild ($\alpha\beta^R$) or from HbS ($\alpha\beta^S$) in the presence of β_4 (HbH). In either exchange, $\alpha_2\beta_2$ (HbA) is formed. (C) Dissociation rates for the dimer−monomer interface. Note that for weak hemoglobins the exchange is on a per hour basis and for strong hemoglobins it is on a per day basis. Since the assay measures adult HbA, the value of the $\alpha\beta$ dimer itself cannot be obtained so sickle Hb ($\alpha\beta^S$) was used.

calculated from their dissociation constants are shown in Figure 2A. These thermodynamic values are also a measure of tetramer stability (red arrow) and show an undulating pattern similar to the developmental profile in Figure 1B. However, there is no apparent correlation of the values themselves with the time of expression (Figure 1B) or gene order (Figure 1C). For example, the free energy values of embryonic Hb Portland-1 ($\zeta_2\gamma_2$) and of embryonic Hb Gower-2 ($\alpha_2\varepsilon_2$) are equal to and lower than, respectively, that of adult HbA ($\alpha_2\beta_2$) (Figure 2A). However, there is a consistent pattern on the effects of ζ- versus α-subunits on tetramer strength; i.e., the tetramer−dimer interface is weakened by more than 1 order of magnitude for hemoglobins containing ζ-subunits compared to corresponding tetramers containing α-subunits, representing a free energy increase of 2−7 kcal/mol (Figure 2A). To put this free energy change in more familiar terms, a free energy difference of 6 kcal/mol exists between deoxy $\alpha_2\beta_2$ and oxy $\alpha_2\beta_2$ (−14 and −8 kcal/mol, respectively) (*13*), arising from the more extensive subunit interactions in deoxy $\alpha_2\beta_2$.

Relationship between Equilibrium States. The results in Figure 2A suggest that some hemoglobins form tetramers from dimers more efficiently than others (*16*). Thus, tetrameric fetal HbF ($\alpha_2\gamma_2$) is formed at very low Hb concentrations because it has the lowest tetramer−dimer free energy (*17*) (Figure 2A). This is consistent with the rapid increase in the level of fetal HbF in the fetal period of development shown in Figure 1B (depicted as γ) compared to the lag in adult HbA (depicted as β) during this period. Hence, the intrinsic differences in subunit interface strengths of HbF and HbA bestow the ability to favor formation of one Hb over the other in a young maturing erythrocyte at low Hb concentrations. To measure the magnitude of the effect of the tetrameric state on dimers of the $\alpha\beta$ type, we compared the subunit exchange rates of two natural mutants, i.e., dimeric Hb Rothschild ($\alpha\beta^R$) and tetrameric sickle HbS ($\alpha\beta^S$) (Figure 2B); Hb Rothschild cannot form tetramers because of the mutant amino acid substitution in the tetramer−dimer interface (*18*). Since these two hemoglobins have the same dimer−monomer interfaces, the effect of the tetrameric state of HbS on the amount of dimer present can be distinguished. Dimeric Hb Rothschild dissociated to monomers ~3 times faster than tetrameric sickle HbS did (Figure 2B), showing that the presence of tetramer rapidly displaces the overall monomer−dimer−tetramer equilibrium to the right (Figure 2B caption), resulting in less dimer available for dissociation to monomers and subsequent exchange with normal β-subunits to form adult $\alpha_2\beta_2$.

Dimer−Monomer Interface. To estimate the strength of this interface, we used the observation of Huehns et al. (*19*) that exchange of subunits between two different hemoglobins occurs at low pH due to dissociation of their respective dimers followed by re-equilibration and association of the resultant monomers to form other dimer types. After modifying this method for human hemoglobins containing either ζ- or α-subunits (*16*), we could obtain estimates of their dissociation rates. The results in Figure 2C show the overall range in these rates plotted in the order of dimer dissociation for seven of the eight normal human hemoglobins. Embryonic hemoglobin dimers are the weakest, fetal hemoglobins stronger, and adult hemoglobins the strongest (progression of strength indicated by the vertical red arrow in Figure 2C). Hemoglobins containing ζ-subunits dissociate to monomers much more readily than do those containing α-subunits. Dimers with α-subunits have increasing strengths that correlate with the gene order of the β-like subunits (ε, γ, δ, and β),

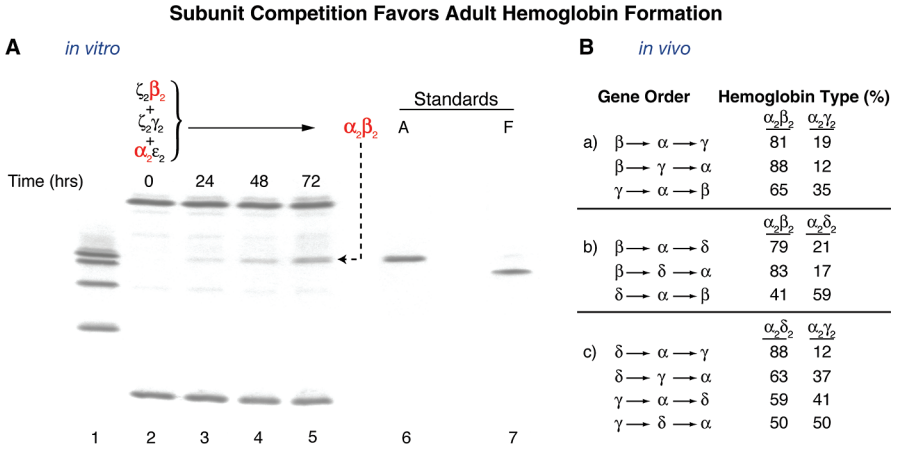

FIGURE 3: (A) In vitro subunit competition. Hemoglobins Portland-2 ($\zeta_2\beta_2$), Portland-1 ($\zeta_2\gamma_2$), and Gower-2 ($\alpha_2\varepsilon_2$) (10 μM each) were mixed, and the amount of HbA ($\alpha_2\beta_2$) formed was measured by isoelectric focusing after 1, 2, and 3 days. No HbF ($\alpha_2\gamma_2$) was detectable. (B) In vivo subunit competition. The order of the α-, γ-, δ-, and β-genes was varied in the in vivo yeast expression system. Each Hb expressed was measured by FPLC and quantitated as described in the text.

whereas dimers containing ζ-subunits have strengths of reverse gene order. The two weakest hemoglobins, $\zeta_2\beta_2$ and $\zeta_2\delta_2$, are also the most rare; they are found only in extreme cases of homozygous α-thalassemia ("hydrops fetalis") where synthesis of the α-subunit is absent (*20*). This is likely because these dimers have the weakest interface strength of all human hemoglobins. The wide range of subunit interface energetics displayed in Figure 2C has not previously been appreciated and suggests a linkage between their energetic levels and their developmental profile. These features are reminiscent of the HOX proteins that are responsible for morphological development and are also expressed in a specific temporal order (see Discussion).

Interface Subunit Bonding Strengths Correlate with Transitions in Their Developmental Profile. The results given above offer an explanation for the developmental profile in Figure 1B due simply to the subunit interface properties of the hemoglobins themselves. The three embryonic hemoglobins, $\zeta_2\varepsilon_2$, $\zeta_2\gamma_2$, and $\alpha_2\varepsilon_2$, (shown as monomeric ζ, γ, and ε, respectively, in Figure 1B) disappear rapidly during the prenatal period most likely because they have the weakest dimer−monomer interface strengths (Figure 2C). The rapid increase in the level of $\alpha_2\gamma_2$ (HbF) in the early prenatal phase and the much slower increase in the level of $\alpha_2\beta_2$ (HbA) (represented by "β" in Figure 1B) are consistent with the HbF having a tetramer−dimer free energy lower than that of HbA; both have strong dimer−monomer interfaces (Figure 2C). Thus, there is an energetic barrier causing a lag in adult $\alpha_2\beta_2$ production (Figure 1B). The decrease in the level of fetal $\alpha_2\gamma_2$ just prior to birth could be due to the slow post-translational acetylation of γ-subunits to produce $\alpha_2\gamma_2^{Ac}$, which has subunit strengths close to that of HbA (*21*), thus annulling the effect of the low free energy of HbF. HbA_2, $\alpha_2\delta_2$ (shown as δ in Figure 1B), is present at very low concentrations in adult blood; the αδ dimer, unlike other hemoglobin dimers, is weak as indicated by its inordinately large peak width during gel filtration, suggesting that it exists in a facile equilibrium with its monomers thus limiting its ability to form tetramers efficiently (*22*). HbA_2 exchanges with β-subunits (in the form of β_4) very slowly (bottom line in Figure 2C).

Competition during Subunit Exchange. Embryonic red cells contain several hemoglobins having relatively weak subunit interfaces (Figure 2C). Since these hemoglobins are present simultaneously in these young red cells, we tested whether, when mixed together in vitro, they would dissociate to monomers and reassociate competitively to form different hemoglobins having lower free energy levels and thus stronger interfaces than any of the initial hemoglobins. Equivalent amounts of embryonic hemoglobins $\zeta_2\beta_2$ (Hb Portland-2), $\zeta_2\gamma_2$ (Hb Portland-1), and $\alpha_2\varepsilon_2$ (Hb Gower-2), each of which is in the top part of Figure 2C, were mixed and samples analyzed over time (Figure 3A). The predominant Hb formed was adult $\alpha_2\beta_2$ (HbA). Since α-subunits were present initially in a 1:2 ratio with respect to β- and γ-subunits, the latter subunits would compete with α-subunits to find the more favorable partner. Since no $\alpha_2\gamma_2$ (HbF) was detected, α-subunits favor β-subunits over γ-subunits (see Figure 3A, lane 1 with Hb standards A, F, S, and C from top to bottom and lanes 6 and 7 with HbA and HbF standards, respectively). The subunit exchange results in Figure 3A can be summarized by the equations below and represent a gain of function since the new Hb formed, $\alpha_2\beta_2$, has a lower oxygen affinity and a greater response to allosteric regulators than any of the three initial hemoglobins; these are essential properties for adult Hb. However, we have no evidence that such subunit exchange occurs in vivo.

$$\zeta\beta \rightarrow \zeta + \boldsymbol{\beta}$$

$$\zeta\gamma \rightarrow \zeta + \gamma$$

$$\alpha\varepsilon \rightarrow \boldsymbol{\alpha} + \varepsilon$$

$$\boldsymbol{\alpha} + \boldsymbol{\beta} \rightarrow \boldsymbol{\alpha\beta}$$

Hemoglobin Subunit Competition in Vivo. We extended the in vitro results in Figure 3A by an in vivo study using a yeast expression system constructed with various combinations of one α-gene and a pair of β-, γ-, or δ-genes in a 1:2 ratio in different orders. This approach permitted us to address the relative contributions of gene order and subunit competition to the relative amount of each hemoglobin formed. The yeast expression system that we used (*23*) consists of a small circular DNA plasmid containing galactose promoters; it does not contain a locus control region (LCR). As demonstrated by Wagenbach et al. (*23*) for normal adult HbA and by us (*24*) for mutant sickle HbS, a soluble hemoglobin tetramer is produced with the exact sequence and functional properties of hemoglobin from human

red cells; hemoglobins are expressed in high yield (3−5% of total cellular protein) and include post-translational modifications such as acetylation (*25*) found in natural hemoglobins. Constructs containing α:$\beta + \gamma$, α:$\beta + \delta$, or α:$\delta + \gamma$ genes in various orders were prepared by excising each gene with the appropriate restriction enzyme from the plasmid for $\alpha_2\beta_2$, $\alpha_2\gamma_2$, or $\alpha_2\delta_2$, respectively, prepared previously (*17*, *24*, *26*). These genes were then reinserted in various orders into a fresh plasmid. Sequences and the order of the genes were confirmed prior to induction and expression of hemoglobins, which were separated by FPLC on Mono S and quantitated by weighing cut-out copies of the recorder tracing. The results (Figure 3B, part a) show that α-subunits have a clear preference for β-subunits over either γ- or δ-subunits even when γ is expressed first and β is expressed last, although the relative amount of $\alpha_2\beta_2$ was greater when the β-gene was in the first position. Hence, gene order plays a role along with subunit interface strength in dictating the proportion of HbA and HbF. When δ- and β-subunits were present with α-subunits (Figure 3B, part b), there is a preference for β- over δ-subunits in competing for α-subunits; the only instance in which $\alpha_2\delta_2$ is favored over $\alpha_2\beta_2$ is when β is in the third position and δ is in the first position. Comparing the third constructs in parts a and b, adult δ is a stronger partner for α than fetal γ is. This conclusion was tested in part c of Figure 3B, where δ and γ were allowed to compete directly for the limiting amount of the α-gene. With three of four combinations, $\alpha_2\delta_2$ production prevailed over $\alpha_2\gamma$. Overall, these results are in complete accordance with the energetic order in Figure 2C and reinforce the conclusions about relative subunit interface strengths and their relationship to their development profile. Thus, competition among subunits due to the energetic differences of the dimer interfaces between their monomers and gene order play complementary roles in determining which subunits are paired with more efficiency than others.

DISCUSSION

Subunit Interface Strengths and Gene Order. The order of the dimer−monomer interface strength ($\zeta\beta < \zeta\gamma < \zeta\varepsilon < \alpha\varepsilon < \alpha\gamma < \alpha\delta < \alpha\beta$) is indicative of the decreasing free energy of the interface of each pair (Figure 2C). Our results on the strength of the $\alpha\gamma$-dimer interface (Figure 2C) contrast with those of Mrabet et al. (*27*), who stated that the $\alpha\gamma$-dimer interface was 3 times stronger than the $\alpha\beta$-dimer interface. However, these investigators based their calculations on the erroneous report that HbF ($\alpha_2\gamma_2$) and HbA ($\alpha_2\beta_2$) have the same tetramer−dimer equilibrium constant; however, see refs *16* and *17* and Figure 2A, which show them to differ by nearly 100-fold. Furthermore, the results of Adachi et al. (*28*, *29*) also suggest that the $\alpha\beta$-dimer is stronger than the $\alpha\gamma$-dimer. The $\alpha\beta$-pair has one of the strongest interfaces, but the $\zeta\beta$-pair has the weakest (Figure 2C). On the other hand, the $\zeta\varepsilon$- and $\alpha\varepsilon$-pairs have interface strengths that are much closer to one another (Figure 2C). A possible structural reason for this reciprocal relationship is that ζ- and α-subunits have different degrees of complementarity for their partner subunits; i.e., the α-subunit fits well with the β-subunit but not with the ε-subunit. The opposite is true for the ζ-subunit, which binds tighter to the ε-subunit than to the β-subunit indicative of a reciprocal complementarity. Monomeric subunits alone are known to be unstable (*28*) but are stabilized by dimer formation with partner subunits, although to varying extents as described above.

Analogy between Hemoglobins and HOX Proteins. In the developmental profile of the HOX proteins, there is a correspondence between the order of the HOX genes and the body segments that they express over the course of time in organisms as diverse as *Drosophila* and mice (*30*, *31*). This principle is known as colinearity (*31*), and its molecular basis is a progressive increase in the bonding strength of the various HOX proteins with the DNA to which they bind (*30*). Likewise, with the hemoglobin family, there is a correlation between the increasing subunit interface strength of subunit pairs and the developmental periods during which they occur. Thus, the driving force in both systems is the energetic changes in the bonding strength at subunit interfaces as development proceeds, whether it be with DNA or with a protein partner subunit. It is likely that this theme is repeated often in biological systems.

Subunit Exchange. The results in Figure 3A show that there is an energetic component leading to competition in which α-subunits are favored over ζ-subunits in their ability to combine with partner subunits. In the earlier studies, animal hemoglobins containing α-subunits and various types of β-subunits were used (*19*), so there was no opportunity to detect differences in the energetic levels of the subunit interfaces. Another factor that is likely to play a role in subunit exchange is the self-assembly of some like subunits; e.g., γ-subunits form γ_2 dimers and γ_4 tetramers, as pointed out by Adachi (*28*), and by Kawamura-Konishi and Suzuki (*32*), thereby limiting the amount of monomeric γ-subunits available to combine with α-subunits to form HbF, thus favoring HbA formation. On the other hand, the self-assembly undergone by β-subunits includes β-monomers as part of the equilibrium (*33*, *34*), so free β-subunits are not limiting.

Oxygen Affinity. The adult form of tetrameric hemoglobin has long been a major paradigm for structure−function relationships in proteins. The cooperative binding and release of O_2 and the effect of allosteric effectors (2,3-DPG, H^+, and Cl^-) on this property can be explained in large measure for adult HbA at the structural level (*10*, *17*). An increased level of O_2 binding and weakened responses to allosteric regulators of the embryonic and fetal hemoglobins compared to adult hemoglobin, which has the most favorable binding of 2,3-DPG, and hence lowered O_2 affinity, provide crucial advantages at various stages of development. Attempts to explain differences in O_2 binding and response to regulatory molecules for embryonic and fetal hemoglobins have been made within the framework of the adult Hb structure based on the nature of the amino acid substitutions involved (*9*). Implicit in such homology modeling is the assumption that there are no fundamental differences between these other hemoglobins and adult HbA in their structural features. However, as shown above, there are fairly large differences in the bonding strengths of their subunit interfaces even though their overall architectures are quite similar. In addition, the progressive lowering of the intrinsic oxygen affinity and strengthening response to allosteric regulators from the embryonic to fetal and finally to adult hemoglobins (*9*) closely parallel the changes in the dimer−monomer interface properties shown in Figure 2C. Thus, there also appears to be a linkage between the O_2-related functional properties and the structural properties of this interface.

Role of Acetylation. This post-translational modification of proteins is one of the most common. It is involved in the control of gene expression through modification of ε-NH_2 groups of some Lys residues of histones (*35*, *36*), which form an integral part of the nucleosome structure that represses DNA transcription. Some globin subunits are also acetylated, i.e., the N-terminal Ser of the ζ-subunits of the embryonic hemoglobins

and the N-terminal Gly of the γ-subunit of fetal hemoglobin (HbF_1). We found that natural acetylated HbF_1 and recombinant hemoglobins with acetylated Ser expressed in yeast have weakened subunit interfaces (*21*, *25*). Thus, acetylation may be involved in the developmental progression ("switch") from expression of HbF with strong subunit interfaces to HbA with weaker subunit interfaces mediated by acetylated HbF_1, which also has weakened subunit interfaces. This mechanism may account for part of the developmental progression (switch) of the γ-subunits of HbF to the β-subunits of HbA (Figure 1B). Such a mechanism may also be operative in specific histone acetylation to loosen the nucleosome structure leading to gene expression (*36*).

Current Models of Control of Globin Expression. The prevailing model (*8*) for control of gene expression of the α-like globin gene cluster (ζ-, α^2-, and α^1-genes) and the β-like globin gene cluster (ε-, γ^G-, γ^A-, δ-, and β- genes) suggests that regions upstream from the embryonic ζ- and ε-loci control their expression in a sequential fashion (see Figure 1B) after the chromatin regions encasing these genes are opened, thereby exposing promoter and enhancer regions. These control regions are called the upstream region element (α-URE) for the α-like globin gene cluster and the locus control region (β-LCR) for the β-like globin gene cluster. The globin proteins themselves have not been considered to have an active role in the process perhaps because it has been assumed that they have similar subunit interface strengths. The location of the embryonic ζ- and ε-globin genes adjacent to the upstream regulatory regions is thought to be necessary to ensure that embryonic hemoglobin expression occurs prior to fetal Hb and adult Hb expression for normal hematopoiesis to take place. However, a number of observations are difficult to explain with this model alone. For example, the report by Leder et al. on the simultaneous presence of mRNAs for both ζ- and α-subunits (*37*) and also their finding that deletion of the ζ-gene in mice had only minor consequences on their survival (*38*) seem inconsistent with this model. Indeed, the earlier reports of Wong et al. (*39*) about the occurrence of adult hemoglobin in addition to embryonic hemoglobins in early mouse embryos support the findings of Leder et al. The normal order of genes in both the α- and β-globin gene clusters in relation to the upstream regulatory regions (Figure 1C) has been considered to be of primary importance for the correct temporal expression of the embryonic, fetal, and adult hemoglobins (Figure 1B). However, the results of the gene inversion studies of Tang et al. (*40*) and of Tanimoto et al. (*41*) showed that when the embryonic genes were moved to the most distant positions from the upstream regulatory regions, they were thereby rendered transcriptionally silent but the adult genes were expressed at all stages. The reasons given for these findings were based upon the presumed disruption of interactions with promoters within the upstream regulatory regions. In our opinion, these results are consistent with the relative strengths of the subunit pairings; i.e., the progression from higher to lower free energy allows for normal development of the pathway having normal gene order (shown by the red arrow in Figure 2C). When gene order is reversed with adult α- and β-genes in the proximal positions, its low free energy would create an energetic barrier against formation of the weaker embryonic subunit pairs with higher free energies. In other words, the driving force in the normal progression of the formation of subunit pairs is the continuous lowering of the free energy level at each step; if that order is reversed, the weaker (higher energetic level) hemoglobins will not

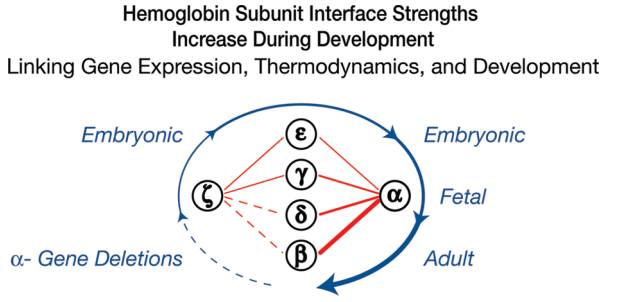

FIGURE 4: Inter-relationship between expressed hemoglobin subunits (black Greek letters), the strength between subunit pairs (red line width representing an increased level of bonding), and the normal temporal formation of hemoglobin types [shown with a solid blue line whose width is developmental progression over time (arrows); the dashed blue line indicates very weak ζβ- and ζδ-pairs present only when all α-genes are inactivated].

be formed. Finally, Randhawa et al. (*20*), who found that $\zeta_2\beta_2$ is formed only in the complete absence but not in the presence of α-subunits, suggested that there was a higher affinity of β-subunits for α-subunits compared to ζ-subunits. This concept of variable affinities of globin subunits in their search for partner subunits is a general principal of the progressive development of the normal hemoglobins and likely applies to other protein networks in general.

Conclusions. Globin subunits find partner subunits depending on when they are expressed (gene order) as regulated by transcriptional events and on the strength of the interface between the subunits (free energy). The subunit interfaces of the embryonic hemoglobins are expressed first, and they also have the weakest dimer−monomer interface and therefore have the shortest lifetime. The very low free energy of the subunit interfaces of fetal Hb is increased by its post-translational acetylation, which contributes to the "genetic switch" between Hb types during development. Adult HbA also has strong subunit interfaces but is not subject to acetylation or to the limitation of self-assembly of like subunits incurred by fetal Hb, thus making it the most favored assembly. The free energy changes of the hemoglobin subunit interfaces over time can be considered as an important contributor to its developmental profile together with gene order under control of transcriptional events, suggestive of a genetic−thermodynamic linkage between them (Figure 4).

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