Subunit Assembly of Hemoglobin: An Important Determinant of Hematologic Phenotype

By H. Franklin Bunn

Hemoglobin's physiologic properties depend on the orderly assembly of its subunits in erythropoietic cells. The biosynthesis of α- and β-globin polypeptide chains is normally balanced. Heme rapidly binds to the globin subunit, either during translation or shortly thereafter. The formation of the αβ-dimer is facilitated by electrostatic attraction of a positively charged α-subunit to a negatively charged β-subunit. The αβ-dimer dissociates extremely slowly. The difference between the rate of dissociation of αβ- and αγ-dimers with increasing pH explains the well-known alkaline resistance of Hb F. Two dimers combine to form the functioning α2β2-tetramer. This model of hemoglobin assembly explains the different levels of positively charged and negatively charged mutant hemoglobins that are encountered in heterozygotes and the effect of α-thalassemia and heme deficiency states in modifying the level of the variant hemoglobin as well as Hb A2. Electrostatic interactions also affect the binding of hemoglobin to the cytoplasmic surface of the red cell membrane and may underlie the formation of target cells. Enhanced binding of positively charged variants such as S and C trigger a normally dormant pathway for potassium and water loss. Thus, the positive charge on β is responsible for the two major contributors to the pathogenesis of Hb SC disease: increased proportion of Hb S and increased intracellular hemoglobin concentration. It is likely that electrostatic interactions play an important role in the assembly of a number of other multisubunit macromolecules, including membrane receptors, cytoskeletal proteins, and DNA binding proteins.

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with the challenging task of diffusing within the thick tomato soup and finding unlike partner subunits so that they can assemble into the functional $\alpha_2\beta_2$-tetramer. A growing body of hematologic and experimental evidence indicates that subtle alterations in the rate of subunit assembly are prime determinants of the distribution of hemoglobins seen in normal and pathologic red cells and can explain a number of hitherto puzzling phenotypic features.

The steps in hemoglobin assembly are depicted schematically in Fig 1. Note that a heme-intact $\beta$-subunit will combine with a heme-intact $\alpha$-subunit or, alternatively, may aggregate with like $\beta$-subunits to form $\beta_2$-tetramers or Hb H. Under normal circumstances the concentration of free $\beta$-subunits is likely to be sufficiently low that there is no significant amount of $\beta$ in the cytosol of erythropoietic cells. In contrast, in certain types of $\alpha$-thalassemia, Hb H and its fetal counterpart Hb Barts (\(\gamma_4\)) can be detected, and in the case of hydrops fetalis (deletion of all four $\alpha$-globin genes \(-/\-\)), these homotetramers are virtually the only hemoglobin detected.

The critical and rate-limiting step in hemoglobin assembly is the simple bimolecular reaction $\alpha + \beta \rightarrow \alpha\beta$. This noncovalent reaction is nearly irreversible. As discussed later, the reverse reaction does occur but slowly. In contrast, the combination of two like $\alpha\beta$-dimers to form the $\alpha_2\beta_2$-tetramer is readily reversible. The interface at which these two dimers slide on each other is crucial to the cooperative behavior of hemoglobin.

**SURVEY OF MUTANT HEMOGLOBINS**

The vast body of information on the human hemoglobin variants can provide special insights into the mechanism of subunit assembly. Several independent factors influence the formation of normal and mutant hemoglobins. A few variant subunits are synthesized at a significantly lower rate than that of their normal counterparts. Examples include Hb E$^{4-7}$ and Hb Knossos, in which the mutations lead to defective $\beta$-globin mRNA processing, and therefore significantly reduced protein synthesis and a thalassemic phenotype. In contrast, the vast majority of variant subunits appear to have normal rates of transcription and translation. Another factor that influences the amount of variant hemoglobin in the red cell is the stability of the variant subunit. Among the 70-odd unstable $\beta$-globin variants that have been reported to date, most constitute less than 30% of the total hemoglobin, owing to increased catabolism of the variant both in erythroid precursor cells and in the circulation.

A third factor, differences in the rates of subunit assembly, could explain the variability in the levels of most of the stable hemoglobin variants. Even small differences in the rates of combination of variant subunits with normal partner subunits could have a significant effect on the distribution of hemoglobins in the red cell. In order to explore this hypothesis, we surveyed reports in the literature on $\beta$-globin variants. Humans inherit two $\beta$-globin genes, one from each parent. Accordingly, if a variant $\beta$-subunit is translated at a normal rate and has normal stability and solubility, red cells of heterozygotes would be expected to contain equal amounts of the normal and variant hemoglobin. However, the reported values show unexpected variability. Many of the commonly encountered $\beta$-globin variants are present in significantly lower amounts than in Hb A. As shown in Fig 2, these variants tend to be positively charged. In the presence of $\alpha$-thalassemia, where there is a limiting amount of $\alpha$-chains, the proportion of the positively charged variant is further decreased in proportion to the number of $\alpha$-globin genes deleted. In contrast, negatively charged variants are often present in heterozygote red cells in amounts greater than Hb A. In one case (Hb J-Baltimore), the presence of $\alpha$-thalassemia resulted in an increase in the proportion of the variant. Another variant (Hb N-Baltimore) was unchanged by the presence of $\alpha$-thalassemia.

The levels of the stable $\beta$-globin variants shown in Fig 2 are independent of the location of the amino acid substitution on the surface of the molecule. All substitutions at the interfaces between $\alpha$ and $\beta$ subunits were excluded from this analysis because structural alterations at these sites could have a direct effect on hemoglobin assembly independent of charge. Moreover, $\alpha$-globin variants were excluded for two reasons. First, the levels of $\alpha$-globin mutants are markedly affected by $\alpha$-thalassemia, a condition that is often not well

![Fig 1. Assembly of hemoglobin. The $\alpha$- and $\beta$-globin polypeptides are translated on their respective mRNAs. On binding of heme, the protein folds into its native three-dimensional structure. The binding of $\alpha$- and $\beta$-hemoglobin subunits to each other is facilitated by electrostatic attraction. An unstable intermediate encounter complex can rearrange to form the stable $\alpha\beta$-dimer. Two dimers combine to form the functional $\alpha_2\beta_2$-tetramer.](image)
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ASSOCIATION OF MONOMERS INTO αβ-DIMERS

The rate-limiting step in hemoglobin assembly is the combination of heme-intact α- and β-globin subunits to form the αβ-dimer. This reaction can be monitored by spectroscopic means to give a second-order rate constant of about 8 × 10⁶ M⁻¹ s⁻¹. This rate is nearly three orders of magnitude slower than the diffusion-limited rate for the combination of two macromolecules the size of globin subunits. Because of inherent inaccuracies in these spectroscopic measurements, we used subunit competition experiments to examine the assembly of hemoglobin variants and to test the effect of surface charge. Previous experiments had shown that α-subunits combine about two times more readily with β⁺ than with β⁻.

Our subunit competition experiments were performed by incubating varying amounts of α-subunits with an equimolar amount of normal (β⁺) and variant (β⁻) β-subunits and then measuring the amounts of hemoglobin formed (Hb A and Hb X) by high-performance liquid chromatography and isoelectric focusing. Globin subunits were prepared with sufficient care that the addition of α-chains to an equivalent amount of β-chains resulted in the stoichiometric formation of hemoglobin tetramers having completely normal oxygen binding. These competition experiments closely simulate the in vivo situation in erythroblasts of heterozygotes where normal and variant β-subunits are competing for α-subunits. A low ratio of α to β⁺ + β⁻ mimics the coexistence of α-thalassemia. At physiologic pH (7.2), the α-subunit has 2.4 positive charges, while the β⁻ subunit has 2.5 negative charges. Therefore, it makes sense that the two subunits would be pulled together by electrostatic attraction. This attraction would be altered if a variant β-subunit (β⁻) differed significantly in surface charge from β⁺. As shown in Fig 3, in the presence of limiting amounts of α-chains, the formation of Hb X relative to Hb A was directly related to the surface charge of the variant β-subunit. At pH 8.0, where α-subunits are at their isoelectric point and therefore lack significant net surface charge, differences in charge on the β-subunit no longer affect the rate of αβ-dimer formation. These subunit competition experiments are in satisfactory agreement with a theoretical model based on the effect of smeared charge on the diffusion-limited association of macromolecules. As mentioned earlier, the overall rate of combination of α- and β-subunits is 1,000 times slower than the diffusion-limited reaction rate. Moreover, there is no significant difference in the rate of dissociation of αβ-dimers of widely different charges (αβ⁺, αβ⁻, αβ⁻⁻). Taken together, these observations strongly suggest that hemoglobin assembles by the formation of an intermediate encounter complex (α·β) that can relax into the stable αβ-dimer:

\[ \alpha + \beta \rightarrow \alpha \cdot \beta \rightarrow \alpha \beta. \]
and a variant α-subunit (ft). Data from Mrabet et al.\textsuperscript{15} α\textsubscript{X} and γ\textsubscript{X}, for competitive recombination reactions between α\textsubscript{X} and β\textsubscript{X} (data from Mrabet et al.\textsuperscript{15} ).

A ratio is a measure of the relative rates of assembly of αA and αAβ\textsubscript{S} subunits. At intracellular pH (7.2), the relative rates of α/β-dimer formation is shown in Fig 3, the relative rates of αAβ\textsubscript{S} formation is higher in SC red cells than in AS red cells.\textsuperscript{37,38} The latter finding can be explained by differences in the rates of subunit assembly. As shown in Fig 3, the relative rates of αβ-dimer formation is A > S > C. These differences are reflected in the levels of hemoglobins in AS and SC red cells. The converse situation pertains when Hb S is inherited with a negatively charged β-globin variant. In these double heterozygotes, the proportion of Hb S is even lower, and thus the proportion of the variant is even higher than that found in association with Hb A. For example, the proportion of Hb Pyrgos (β 83 Gly → Asp) was 62% in an S/Pyrgos double heterozygote, compared with 52% in relatives who were A/Pyrgos heterozygotes.\textsuperscript{39} These findings are again fully consistent with the effect of surface charge on the assembly of normal and variant hemoglobins.

Measurement of minor hemoglobin components has been useful in the diagnosis of a number of hematologic disorders. Hb A\textsubscript{2} (α\textsubscript{2}δ\textsubscript{2}) normally constitutes about 2% of the hemolyzate. This component is increased approximately twofold in β-thalassemia trait and decreased in various forms of α-thalassemia\textsuperscript{40} as well as in two acquired disorders: iron deficiency\textsuperscript{41,42} and sideroblastic anemia.\textsuperscript{43} In the latter two conditions, a relative deficiency of α-globin synthesis has been noted in some studies.\textsuperscript{43,44} Therefore, they can be considered acquired forms of α-thalassemia. The δ-globin subunit is considerably more positively charged than is the β-subunit. Therefore, when the production of α-globin is limiting (α-thalassemia), the relative amount of Hb A\textsubscript{2} would be expected to decrease. In contrast, when α-subunits are present in excess, as in β-thalassemia, the small amount of δ-subunits that are produced should be fully titrated, and therefore Hb A\textsubscript{2} increases. This proposal is supported by competition experiments showing that α-subunits bind more avidly to β- than to δ-subunits.\textsuperscript{41} The contribution of subunit competition to the formation of Hb A\textsubscript{2} provides a reasonable explanation for the slight elevation in Hb A\textsubscript{2} that has been noted in individuals with sickle trait (AS)\textsuperscript{45} as well as in SS homozygotes who also have α-thalassemia.\textsuperscript{46}

Hb F (α\textsubscript{2}γ\textsubscript{2}), the major hemoglobin component of the fetus, is normally replaced by Hb A by means of a switching mechanism that takes place during the latter part of gestation. Whatever Hb F that remains is compartmentalized in a restricted population of red cells. Elevations of Hb F in a variety of congenital and acquired disorders are generally proportional to the number of F cells that are produced. However, levels of Hb F could also be affected by differences in assembly. Because β- and γ-subunits differ considerably in primary structure (39 out of 146 residues), it is likely that factors other than charge affect their relative rates of binding to α-subunits. Because native heme-intact γ-subunits are difficult to isolate and work with, no competition experiments have been done. However, some recent clinical observations address this issue. In newborns, the proportion of Hb F is lower in those with α-thalassemia than in those having all four α-globin genes.\textsuperscript{40} In addition, an individual heterozygous for deletion-type hereditary persistence of fetal hemoglobin was found to have only 7% Hb F when he had severe iron deficiency.\textsuperscript{51} Iron replacement resulted in increased synthesis of α-globin subunits as well as an increase in Hb F to a steady-state level of 25%. These hematologic observations suggest that α-subunits combine less readily with γ- than with β-subunits.

In keeping with this model of hemoglobin assembly are the observations that when AS and AE heterozygotes become iron deficient, the level of the positively charged variant hemoglobin drops markedly,\textsuperscript{52,53} but, as with the patient described earlier, is readily corrected by iron replacement. It is likely in all these cases that the acquired α-thalassemia
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intensifies the competition between normal and the variant subunit.

Although a large body of hematologic and experimental observations supports this model certain exceptions can be cited. For example, the level of Hb C in AC individuals is about the same as that of Hb S in those with sickle trait (Fig 2). Moreover, α-thalassemia has no effect on the proportion of S and C hemoglobin in individuals with SC decrease. Thus, in vivo ββ and βC compete about equally well for α-subunits, whereas in vitro (Fig 3) βC combines about three times as readily as ββ. It is possible that mild instability of Hb S55 is an independent contributor to the proportion of Hb S in circulating red cells.

TARGET CELLS

The surface charge of hemoglobin may impact on another phenotypic feature: red cell volume. One of the most commonly observed characteristics of “hemoglobinopathies” is the presence of target cells on dried blood films. This finding is accompanied by a shift to the right of the osmotic fragility curve indicative of a relative decrease in the ratio of cell volume to surface area. Target cells are prominent in the following commonly encountered genotypes: CC, AC, SS, EE, AE, and DD. In a rare homozygote with hemoglobin O-Arab, both target cells and a right-shifted osmotic fragility were noted. All of these variants are positively charged. There is solid experimental evidence that hemoglobin binds to the inner surface of the red cell membrane by electrostatic interactions, probably to a negatively charged domain on the cytoplasmic side of the major integral membrane protein, Band 3. Therefore, it is likely that this binding is affected by the surface charge of hemoglobin. Hb C binds more strongly than Hb A to the cytoplasmic surface of red cell membranes. This “assembly” may be an integral part of a recently described pathway that causes leakage of potassium and water from red cells. This pH-and-ionic-strength-dependent pathway is active in CC red cells and is also readily observed in AC, SS, and EE red cells. Thus, the surface charge of hemoglobin may be a critical determinant of red cell volume and may explain the presence of cell dehydration and target cells that is such a prominent feature of the common positively charged variants.

BROADER IMPLICATIONS

The assembly of hemoglobin is apt to be relatively simple compared with that of other biologically important macromolecules. An array of biophysical probes is becoming available to investigate these important posttranslational events. Examples of particular interest to hemotologists include (a) secreted proteins, such as immunoglobulin and factor VIII-von Willebrand factor; (b) membrane complexes, such as the T cell receptor for antigen and platelet membrane glycoproteins 2B-3A; (c) structural proteins such as the spectrin-actin-4.1 assembly in the red cell cytoskeleton; and (d) the complex variety of protein-DNA and protein-RNA interactions that regulate gene expression.

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