Protein Conformational Heterogeneity as a Binding Catalyst: ESI-MS Study of Hemoglobin H Formation†

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ABSTRACT: Our previous studies of hemoglobin tetramer assembly in vitro suggested that the initial step in the oligomerization process, which ultimately dictates the high fidelity of the heterotetramer $(\alpha^*\beta^*)_2$ assembly, is the binding of a flexible heme-free β-globin chain to a highly ordered heme-bound α^*-globin. In this work, we extend these studies to investigate formation of the homotetrameric hemoglobin H, whose formation in vivo is a well-documented clinical consequence of significant overexpression of β-globin in α-thalassemic disorders. Upon reconstitution of the isolated β-globin with excess heme, the predominant species in the ESI mass spectrum corresponds to the homotetramer β^*_4, alongside homodimeric species and monomeric β-globin chains in both apo and holo forms. The assembly process of the hemoglobin H homotetramer apparently follows a scenario similar to that of a normal heterodimeric hemoglobin $(\alpha^*\beta^*)_2$ species, with the asymmetric binding event between compact and flexible polypeptide chains being the initial step. The extreme importance of large-scale chain dynamics and conformational heterogeneity for the protein assembly process is highlighted by the inability of highly structured α-globins to undergo ordered oligomerization to form dimers and tetramers as opposed to indiscriminate aggregation.

Mammalian hemoglobins (Hbs)[1] are tetrameric proteins that are normally composed of two chains (α- and β-globins in adult organisms to form an $\alpha_2\beta_2$ noncovalent assembly) and constitute a class of biopolymers that remained the focal point of extensive biochemical and biophysical studies in the past several decades (*1*). While most efforts are aimed at a detailed understanding of Hb ligand (dioxygen) binding properties, their modulation by Hb structure, and underlying phenomena of cooperativity and allostery, recent years have witnessed growing interest in the aspects of protein behavior related to high-fidelity assembly of a functional oligomer from its monomeric constituents (*2*−*5*). Despite the fact that the two adult globin chains have a high degree of similarity in terms of both sequence homology (*6*) and tertiary folds (*7*), their roles in the assembly process appear to be remarkably different (*8*). Initially, a highly structured heme-bound α-globin (α^*) interacts with a flexible β-globin chain to form the so-called semi-hemoglobin dimer ($\alpha^*\beta$). The degree of structural disorder within the monomeric β-chains is so significant that they are not able to retain the heme group efficiently, although binding to α^* monomers dramatically reduces their flexibility, apparently "snapping" them into "correct" fold. Thus, formation of semi-hemoglobin endows β-chains with heme binding competency, leading to formation of a "normal" hemoglobin dimer, $\alpha^*\beta^*$, dimerization of which produces a tetrameric Hb species, $(\alpha^*\beta^*)_2$ (*8*).

Although it is quite clear that a natively folded α^*-chain serves as a rigid template for β-globin binding and, therefore, effectively directs the entire Hb assembly process, several questions concerning the roles of the two globins in the binding process still remain unanswered. Despite a significant degree of structural heterogeneity exhibited by monomeric β-globins, in the absence of α-chains they (as well as their fetal analogues, γ-globins) are able to form homotetrameric assemblies in vivo (*9*, *10*), the quaternary structure of which is remarkably similar to that of normal Hb species, $(\alpha^*\beta^*)_2$ (*11*−*13*). This behavior contrasts sharply with that of α-globins, which do not undergo orderly oligomerization to form homodimeric or tetrameric species in the absence of their counterparts and in fact require a specialized chaperone system (*14*) converting excessive free α^* monomers in erythrocytes to chemically inert states (*15*). It seems puzzling that a globin lacking well-defined structure is capable of forming tetrameric species with a nativelike structure, while the more stable polypeptide fails to do so.

In addition to the fundamental importance of finding determinants of orderly protein oligomerization, deciphering the mechanisms of self-association in the Hb system has significant practical ramifications. Indeed, such knowledge would be extremely valuable for the design of Hb-based blood substitutes and managing a variety of pathological conditions linked to abnormalities in the Hb assembly process, especially thalassemias (*16*). Furthermore, the oligomerization state of both wild-type and chemically modified Hb appears to be an important factor dictating the efficiency of the interaction of Hb with haptoglobin, a protein

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[1] Abbreviations: ESI-MS, electrospray ionization mass spectrometry; FTIR, Fourier-transform infrared spectroscopy; Hb, hemoglobin; Hb H, hemoglobin H; HPLC, high-performance liquid chromatography; mRNA, messenger RNA; NMR, nuclear magnetic resonance.

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that sequesters free Hb and removes it from circulation to minimize oxidative damage during hemolysis (*17*).

Although the homotetrameric nature of Hb H was revealed (*10*) soon after the discovery of this abnormal hemoglobin type (*9*), the uniqueness of the β-globins' (as well as the closely related fetal γ-globins) ability to self-assemble in vivo as a result of an imbalance in the production of α- and β-chains was not realized until decades later. In fact, the intuition suggested at the time that "other abnormal hemoglobins or minor components in normal hemoglobin may be built on the scheme α_4 [and] $\alpha_3\beta$" (*10*). Once the uniqueness of the β-type globins' ability to form stable homotetramers became evident, the keys to understanding this phenomenon were sought in their distinct structural features. The availability of crystal structures of both Hb H β_4 (*11*, *12*) and Hb Bart's γ_4 (*13*) homotetramers provided necessary material for such analyses, with the hope that "structural differences...offer clues as to what factors influence their association/dissociation behavior, and ultimately correct Hb self-assembly" (*13*).

Although X-ray protein crystallography provides a wealth of structural information, by definition it is biased toward the static structures of the most stable state(s). Although dramatic recent technological improvements in this field, particularly in cryocrystallography and the recently introduced time-resolved crystallography, allow in many cases the small-scale dynamic events to be observed (*18*, *19*), large-scale dynamics and protein states with high degree of disorder remain out of reach of this technique. Therefore, exclusive reliance on the X-ray data may provide a picture of β-globin self-assembly that is biased toward structural (static) aspects at the expense of dynamic determinants of binding. Despite a strongly negative connotation that was traditionally attached to structural disorder in the past, recent years brought the realization that large-scale conformational dynamics is in many cases a beneficial feature that often facilitates protein interactions within complex cellular networks (*20*, *21*).

Large-scale conformational changes leading to significant changes in protein shape can be easily detected using spectroscopic methods, such as circular dichroism (*22*), fluorescence spectroscopy (*23*), and FTIR (*24*). However, the structural information provided in such measurements is limited to either cumulative changes in the secondary or tertiary structure or specific markers of dynamic events (e.g., changes in the environment of aromatic residues), and it is averaged across the entire protein ensemble, not allowing a distinction to be made among various protein states that may be present in solution at equilibrium. NMR remains the only spectroscopic technique capable of providing a wealth of both structural and dynamic information about proteins. However, despite its spectacular success in the past years, the utility of high-resolution NMR remains limited in many cases by its low tolerance to paramagnetic ligands and cofactors and a practical molecular mass limitation of ca. 30 kDa. Furthermore, NMR analysis also produces structural data averaged across the entire protein ensemble, without making a distinction between various equilibrium protein states.

Electrospray ionization mass spectrometry (ESI-MS) has recently emerged as a powerful biophysical tool capable of providing a wealth of information about both structure and dynamics of proteins and their assemblies (*25*, *26*). It offers several important advantages, which make it a very attractive tool for probing structure and dynamics of biopolymers. ESI-MS greatly outperforms high-field NMR in its ability to handle larger proteins and their complexes [the practical upper mass limit of ESI-MS is yet to be established, as the bar is being continuously raised (*27*)]. Furthermore, several ESI-MS-based experimental strategies allow protein characterization to be carried out in a conformer-specific fashion, allowing a clear distinction to be made between various conformers of the same protein. Finally, ESI-MS is very successful in addressing a serious problem inherent to all "traditional" biophysical tools, namely, the great difficulty associated with the analysis of protein structure and dynamics in heterogeneous systems. Indeed, ionic signals from different species in multicomponent systems (e.g., solutions containing mixtures of several proteins, as well as other biopolymers) do not generally overlap. Even the overlapping ion peaks can be resolved in many cases on the basis of the difference in their charge states (*28*) or exact masses (*29*).

The aim of this work was to evaluate the role of structural disorder in β-globin self-association in vitro by analyzing protein ion charge state distributions in electrospray ionization (ESI) mass spectra. This technique is a powerful tool for probing dynamic behavior of proteins under a variety of conditions, which has also been used in the past to study assembly and dissociation of Hb heterotetramers (*8*, *30*). Here we demonstrate that it is the conformational heterogeneity of monomeric β-globins in solution that makes the self-assembly process possible, as this protein populates both compact nativelike structures and partially unfolded polypeptide chains under equilibrium. The former provides a binding template, to which the latter adapts, forming initially a homodimeric and, subsequently, homotetrameric species. Elimination of either state effectively inhibits the self-assembly process.

EXPERIMENTAL PROCEDURES

Materials. Hemin chloride and bovine hemoglobin were purchased as lyophilized powders from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals, buffers, and solvents were analytical grade or better.

Preparation of Apo-Hb. Apo-Hb was prepared using a procedure based on acetone precipitation method developed by Antonini et al. (*31*). Specifically, small aliquots of a 1 mM cold (4 °C) aqueous Hb solution were added to cold acetone (−20 °C) containing 3 mL of 2 N HCl per liter of solvent, with vigorous stirring. The final proportions were 1 volume of Hb to 20−30 volumes of acetone. The solution was kept at −20 °C for approximately 20 min with occasional stirring. A pellet of the almost colorless globin precipitate was collected by centrifugation and decantation of the red acetone solution. If residual color remained in the globin precipitate, it was suspended in an acid/acetone solution cooled at −20 °C and separated once more by centrifugation and decantation. The precipitate was redissolved in the minimum required volume of 50 mM ammonium acetate solution and lyophilized.

Liquid Chromatography. Globin chain separation was performed by reversed phase HPLC. A solution of apo-Hb was applied on a C8 analytical column (Agilent Eclipse XDB-C*, 4.6 mm × 150 mm, 5 μm). Globin separation was

obtained using a linear acid gradient from 100% solvent A [45% acetonitrile in 0.3% trifluoroacetic acid (TFA)] to 100% solvent B (45% acetonitrile) over a period of 30 min, at a flow rate of 1 mL/min. The absorbance was measured at 280 nm. Peak fractions were collected and pooled for a large number of injections. The identities of the α- and *β*-chains were confirmed by ESI-MS molecular mass measurement. The pooled fractions were lyophilized to dryness.

Reconstitution of Globin with Heme. Organic solvents, such as acetone and alcohols, had to be removed or their concentrations minimized to prevent unwanted denaturation of the hemoglobin chains. The isolated globin chain stock solutions were prepared by redissolving the lyophilized protein in water to a concentration of 200 *μ*M. A supersaturated heme solution was prepared by mixing hemin chloride and ethanol in a glass vial followed by sonication for ca. 4 h. This supersaturated heme solution was added to the isolated globin solutions in a 1:10 ratio by volume of heme to protein solution. The heme-reconstituted globin solutions were allowed to equilibrate at room temperature for at least 1 h prior to dilution to prepare the samples for final analysis. Upon dilution to the running sample concentration of 10 *μ*M globin, the residual volume of ethanol was less than 1% by volume. The heme:protein molar ratio in the final solution was estimated to be 0.96:1 on the basis of Soret band absorbance measurements and a value for the extinction coefficient (ϵ_{418}) for heme-containing proteins of 191.5 mM^{-1} cm^{-1} reported by Nygaard and co-workers (*32*).

Mass Spectrometry. An Esquire-LC quadrupole ion trap mass spectrometer equipped with a standard ESI source (Bruker Daltonics, Inc., Billerica, MA) was used to confirm the identities of the single globin chains, which were separated by reversed phase HPLC. The fractions were analyzed offline as is, after elution from the HPLC system. All other mass spectra were acquired on a JMS-700 MStation (JEOL, Tokyo, Japan) two-sector mass spectrometer equipped with a standard ESI source. The globin sample solutions were prepared by diluting the stock solution in a 10 mM ammonium acetate solution whose pH was adjusted to a desired level with NH_4OH or CH_3CO_2H. The final protein concentration in each sample was 10 *μ*M. All solutions were equilibrated at room temperature (24 °C) for 1 h prior to analysis. The appearance of the mass spectra did not change when longer equilibration times were investigated. All samples were introduced into the ESI source at a flow rate of 3 *μ*L/min. ESI source settings were used as described previously (*8*), including an orifice potential of 0 V, a ring lens potential of 55 V, an orifice temperature of 120 °C, and a desolvation plate temperature of 80 °C. These source settings were kept constant throughout all measurements to avoid any possible changes in the ion desorption and transmission conditions. All spectra were acquired by scanning the magnet at a rate of 5 s/decade. Five hundred scans were averaged to record each spectrum to ensure a high signal-to-noise ratio. ESI mass spectra were processed (peak integration) using Microcal Origin (Microcal Software, Inc., Northampton, MA).

RESULTS

Chain Isolation. The reversed phase HPLC chromatogram for apo-bovine hemoglobin is shown in Figure 1A. The

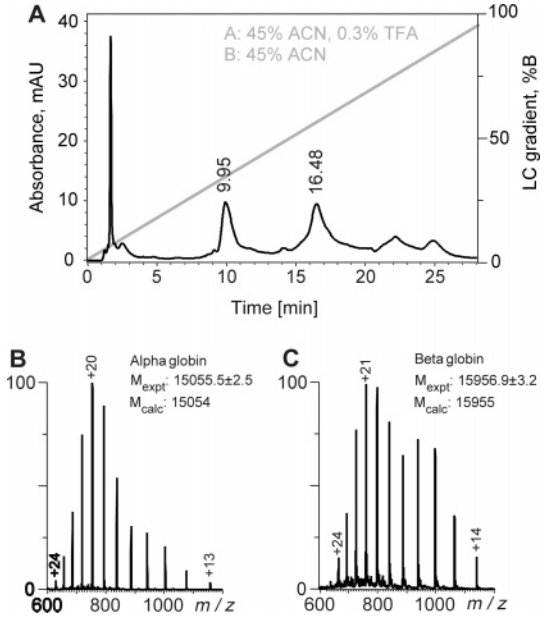

FIGURE 1: Separation of globin chains from bovine Hb with reversed phase HPLC (A). ESI mass spectra of fractions 9−11 (B) and 16−18 (C) confirm the identities of the two species as apo forms of α- and *β*-globins, respectively. The HPLC fractions were collected at a rate of one fraction per minute of elution time. The solvent gradient used to separate the two chains is shown in panel A (gray trace).

α-chain eluted first at approximately 10 min, while the *β*-chain eluted at approximately 16.5 min. A significant elution window between the two globin peaks allowed the facile collection of these fractions without cross contamination. Identification of the two globins was conducted on the basis of the polypeptide masses measured by ESI-MS (Figure 1B,C). The molecular mass of the α-chain was determined to be 15 055.5 ± 2.5 Da (average mass calculated from sequence, 15 053 Da), while the *β*-chain's molecular mass was determined to be 15 956.9 ± 3.2 Da (average mass calculated from sequence, 15 955 Da).

Mass Spectra of Apoglobin Chains under Near-Native Conditions. ESI mass spectra of the isolated apo forms of α- and *β*-globins show that both chains have a highly flexible structure in the absence of the heme group (Figure 2). The two mass spectra are similar to each other, as they display wide distributions of charge states, up to +23. Both charge state distributions are bimodal, revealing the presence of both compact conformers (up to +9) and more disordered (less compact) protein states represented by ion peaks in the lower *m*/*z* regions of the mass spectra (charge states +10 through +23). A notable distinction between the two spectra is due to the presence of a dimer ion signal in the mass spectrum of *β*-globin and the absence of the dimeric species in the spectrum of α-globin. The charge state distribution of the β_2^{+n} ions is rather narrow, hinting at a very low degree of conformational heterogeneity, and is remarkably similar to the distributions of heterodimers observed previously in ESI mass spectra of normal Hb under similar conditions (*8*). No ionic species corresponding to higher-order oligomers (trimers, tetramers, and beyond) were observed in the mass spectra in the *m*/*z* region up to 5200.

Heme Reconstitution of the Isolated α-Globin Chains. ESI mass spectra of isolated α-globin chains after equilibration with a stoichiometric excess of heme in the pH range of >5

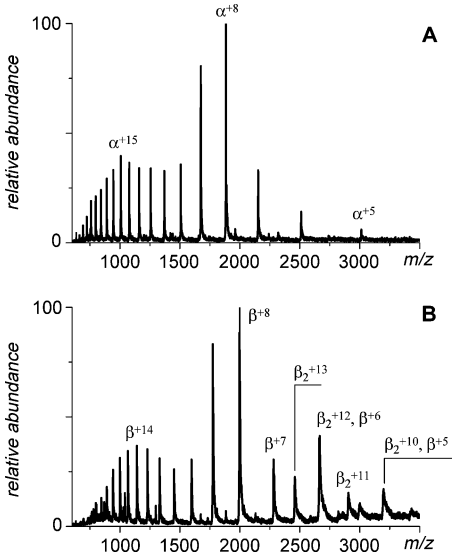

FIGURE 2: ESI mass spectra of 10 μM aqueous solutions [10 mM ammonium acetate (pH 8)] of heme-free α-globin (A) and β-globin (B) isolated from bovine Hb.

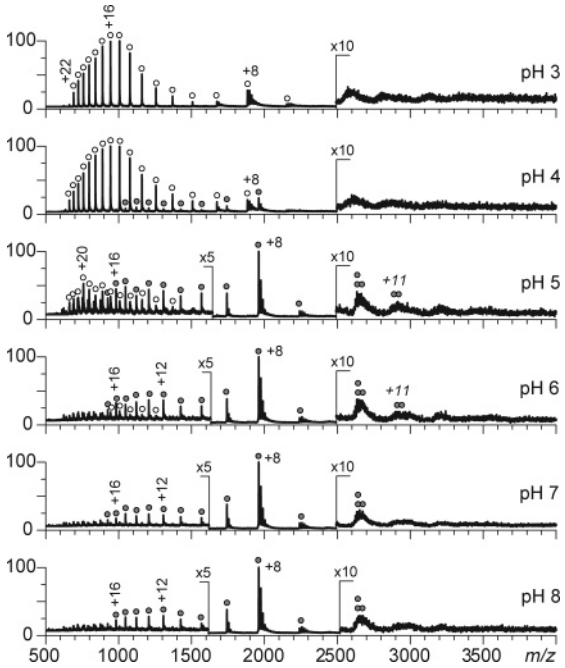

FIGURE 3: ESI mass spectra of 10 μM bovine Hb α-chain reconstituted with a stoichiometric excess of a heme group in 10 mM ammonium acetate arranged in order of increasing pH (from pH 3 to 8). Holoprotein and apoprotein ions are labeled with filled and empty circles, respectively.

show that heme binds to the protein at a 1:1 ratio (Figure 3). Upon reconstitution with the heme group, the α-chain, whose apo form had a significant contribution from flexible conformers (vide supra), apparently folds and becomes locked in a compact (presumably native) state. Behavior of the isolated heme-bound α-chain revealed by ESI-MS is very similar to that of the α-globin monomer in the diluted solution of the hemoglobin heterodimer (*8*). The native conformation remains predominant at pH ≥5. The protein becomes destabilized and loses its heme group under more acidic conditions (Figure 3). A very small fraction of the protein populates non-native states (represented by the higher charge charge density ions, +10 through +17) even at neutral

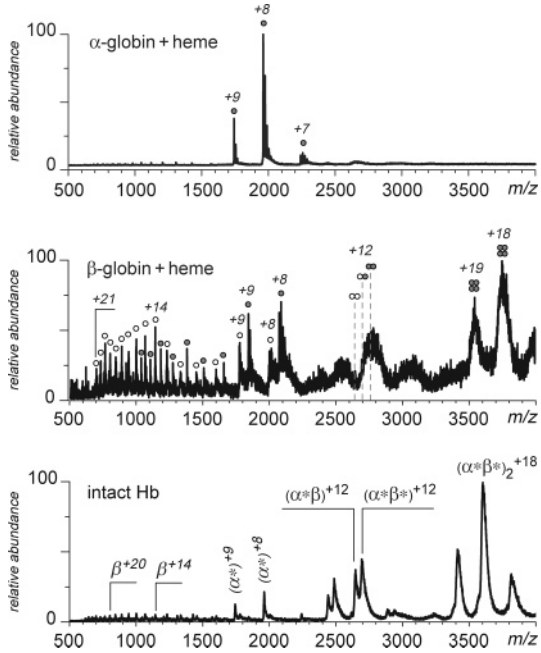

FIGURE 4: ESI mass spectra of the isolated α-chains (top trace) and β-chains (middle trace) of bovine Hb saturated with a large molar excess of a heme group. The mass spectrum of intact bovine Hb (bottom trace) is included for comparison. Calculated *m*/*z* values for putative dimeric ions $(\beta\beta)^{+12}$, $(\beta^*\beta)^{+12}$, and $(\beta^*)_2^{+12}$ are indicated with dotted lines in the middle trace.

and mildly acidic pH. Intriguingly, this apparent partial unfolding does not necessarily result in heme dissociation from the polypeptide chain. Deconvolution of charge state distributions of both heme-bound and heme-free α-globin monomer ions in ESI-MS using a chemometric procedure developed previously in our laboratory (*33*) reveals the existence of at least four different protein states over the entire pH range that was tested (see the Supporting Information for more detail). The ionic signal of α-globin dimers is very weak throughout the entire range of conditions employed in this work and most likely results from nonspecific association during the ESI process (*34*). The extent of dimerization in this case is negligible compared to the yield of heterodimers formed upon mixing isolated α- and β-globins in the presence of heme (see the Supporting Information for more detail). Higher-order oligomers of α-globins were not observed under any conditions used in our work, suggesting that the efficiency of α-globin self-assembly is indeed very low compared to that of heterotetrameric Hb species and β-globin homotetramers (vide infra).

Heme Reconstitution of the Isolated β-Globin Chains. While the heme-reconstituted α-chains in neutral aqueous solutions are monomeric and give rise to a charge state distribution typical for tightly folded (compact) conformations, isolated β-chains mixed with an excessive amount of heme molecules exhibit both conformational and structural heterogeneity. In addition to the β-globin monomers (both apo and holo forms), the ESI mass spectrum acquired at near-physiological pH reveals the presence of abundant dimeric and tetrameric species (Figure 4). Accurate mass assignment for the β-globin oligomers was difficult due to the extensive solvation of the protein ions in the high-*m*/*z* region of the mass spectrum, and attempts to induce thermal or collisional desolvation in the ESI source caused dissociation of the oligomer ions in the gas phase. Nevertheless, careful analysis

of ESI-MS data suggests that both $\beta^*\beta$ and β^*_2 contribute to the dimeric ion signal, while only a heme-saturated oligomer (β^*_4) gives rise to the tetrameric ion signal.

The charge state distribution of β^*_4 ions is nearly identical to that of a heterotetrameric species, $(\alpha^*\beta^*)_2$, in the mass spectrum of normal Hb (compare the middle and the bottom traces in Figure 4). Likewise, the combined charge state distribution of the homodimeric species (containing unresolved contributions from both $\beta^*\beta$ and β^*_2) is very similar to a combined distribution of $\alpha^*\beta^*$ and $\alpha^*\beta$ dimers in the mass spectrum of normal Hb.

All oligomeric ions' charge state distributions are narrow (indicative of a relatively low degree of conformational flexibility of polypeptide chains within these complexes). Contrary to this, charge state distributions of both heme-free and heme-bound monomeric β-globin ions are bimodal, indicating the presence of both compact and disordered states under equilibrium. While heme binding clearly stabilizes the protein, as suggested by the obvious bias of β^* charge state distributions toward the low-charge density ions, apo chains exhibit a significantly higher degree of structural disorder (Figure 4).

DISCUSSION

Assembly of Hb tetramers in a crowded environment of red blood cells produces functional tetrameric $(\alpha^*\beta^*)_2$ assemblies with a surprisingly high fidelity, given the degree of similarity of the two participating globins and the fact that unlike many other multichain proteins, α- and β-globins are not initially expressed as a single preprotein, which is then proteolyzed to produce the end product polypeptides. Although the two adult mammalian globins are encoded by genes located on different chromosomes and are, therefore, synthesized independently, the production "mismatches" occur only under conditions that dramatically suppress expression of α-globins (α:β mRNA ratio of <0.5), leading to the so-called Hb H disease (*35*). The homotetrameric Hb H (and a related fetal Hb Bart's) remain the only two examples of incorrect Hb assembly, leading to production of dysfunctional high-oxygen affinity Hb molecules unable to deliver significant amounts of dioxygen from blood to tissues (*35*). The α-globins are unable to oligomerize in an orderly fashion and in fact require a specialized chaperone-like system controlling the levels of monomeric α-chains under conditions that suppress production of their counterparts (*14*). Although thalassemias are human diseases, imbalance in globin chain expression in other mammals also leads to thalassemic conditions (*36*). Apart from specific pathological manifestations, this also includes formation of homotetrameric β_4 (analogue of human Hb H) (*37*, *38*) and Y_4 (analogue of human fetal Hb Bart's γ_4) species (*39*), but not α_4 species.

Previously, we discovered an important molecular mechanism that directs Hb assembly along the correct route by favoring the $\alpha-\beta$ binding at the expense of other possible types of globin dimerization, such as $\alpha-\alpha$ and $\beta-\beta$ binding (*8*). While most documented binding preferences are controlled structurally, e.g., by means of highly complementary surfaces at the binding interface, the hetero bias in Hb assembly is controlled both structurally and dynamically. The tightly folded α-globin serves as a rigid template, to which a highly flexible β-globin adapts, locking in the conformation that allows it to acquire a heme group. Thus, the initial binding event between ordered and disordered partners leads to formation of a highly structured heterodimer, which possesses a self-complementary surface required for the next step in Hb tetramer formation. Although a recent study suggests that semi-hemoglobin dimer may become a kinetic trap in the presence of organic solvent (*40*), most existing evidence suggests that it is an obligatory intermediate in formation of the Hb tetramer (*4*, *5*).

The inability of α-globin to form a properly folded homodimer is not surprising, since this event is likely to require at least some structural rearrangement of one of the chains to attain the near-native (i.e., α/β-like) structure at the interface region. On the other hand, the capacity of disordered β-globins to form compact homodimers and tetramers might seem surprising, in light of the apparent importance of a highly structured template for the initial binding step. However, careful analysis of charge state distributions of monomeric ions in the ESI mass spectrum of heme-reconstituted β-globin acquired under near-native conditions indicates that the polypeptide chain actually populates a variety of different conformations, including one whose degree of compactness is the same as that of natively folded globins, such as myoglobin and α-globin (*8*). This conformation is represented by the ionic signal at charge states +7, +8, and +9. It is probably more appropriate to describe the β-globin flexibility not in terms of disorder (lack of structure), but a high degree of conformational heterogeneity, as the polypeptides lacking well-defined structure coexist under equilibrium alongside the highly compact (and most likely folded) state.

Although it may be tempting to call this compact β-globin state "native", such a term would actually be a misnomer in this case, as it is not the only state of the protein populated under native conditions. Instead, we will continue to refer to this state as compact, keeping in mind that it is very likely that its higher-order structure follows a blueprint of a generic globin fold (*41*, *42*). When the protein solution is saturated with heme, this compact conformation has the ability to bind and retain the heme group, although a significant fraction of folded polypeptide chains remains heme-free. It seems logical to assume that this conformer also acts as a "marginal" template for dimerization, providing the requisite scaffold to which a flexible β-chain can adapt. The experimental data presented in this work are consistent with the notion of $\beta-\beta^*$ interaction being the initial binding event in a heme-saturated solution, which triggers formation of a homo analogue of a semi-hemoglobin dimer, $\beta^*\beta$. Just like in the case of semi-hemoglobin, this binding event locks the flexible apo-polypeptide chain in a proper conformation, making it competent to bind a heme group. In a heme-saturated solution, this leads to efficient formation of a homodimer, β^*_2.

It is important to note that the compact conformation of the β-chain can serve as a dimerization template even in the absence of a heme group. Indeed, the ESI mass spectrum of an apo-polypeptide solution (Figure 2B) reveals the presence of a significant population of dimer ions, a feature notably absent in the mass spectrum of isolated apo-α-globins acquired under identical conditions. However, oligomerization of apo-β-globins does not proceed beyond

the dimer formation, suggesting that the presence of the heme group is a requirement for tetramer assembly. The results of earlier studies by Elieser and Wright suggest that the removal of the heme group from myoglobin has the most profound effect on the contiguous region spanning the EF loop, the F helix, the FG loop, and the beginning of the G helix (*43*), and this effect is likely to persist within other globins due to the highly conserved nature of the globin fold. Since both the G helix and the FG loop are crucial elements of the interdimer interfaces in both normal Hb tetramer, homotetrameric Hb H (*11*) and Hb Bart's (*13*), insertion of a heme group into a semi-hemoglobin dimer appears to be an essential component of tetramer formation (*44*), without which a self-complementary surface cannot be stabilized, and consequently, the dimer−dimer interface cannot be formed. This explains the absence of heme-deficient tetramers in the ESI mass spectra of both normal hemoglobin and homotetrameric Hb H (Figure 4), as well as the inability of β_2 apo dimers to continue oligomerization in the absence of heme groups and to form tetrameric β_4 species (Figure 2B).

Unlike β-globins, heme-reconstituted α-chains largely fail to dimerize under near-native conditions (Figure 3). The analysis of protein ion charge state distributions clearly indicates that the protein exists mostly in a compact tightly folded conformation in the pH range of ≥5, which may serve as an excellent binding template but is not sufficiently flexible for efficient self-assembly (vide supra). The small fraction of polypeptides that are less structured (chare states of +10 and above) is likely to lack the requisite flexibility as well, particularly in the EF−F−FG−G region, since they appear to maintain their heme binding capacity. That, as well as their low Boltzmann weight, does not allow them to act as efficient flexible binding partners of compact, highly structured states of α-globins. It is puzzling, however, that α-globin dimerization does not occur even in the absence of the heme group (Figure 2A), despite the fact that the protein in this case populates both compact and unstructured states. It seems likely that the conformational space available to the heme-free α-globins does not allow their easy adaptation to the binding partner, effectively inhibiting formation of the homodimeric species in the absence of heme groups.

The ability of β-globins to undergo ordered oligomerization to form dimers and tetramers both in vitro and in vivo highlights the extreme importance of chain dynamics and conformational heterogeneity for the protein assembly process. Neither stable structure nor extreme flexibility alone appears to be sufficient to drive the ordered oligomerization process forward, while a combination of a highly ordered structural template and a flexible partner does result in efficient dimerization. The benefits of intrinsic structural disorder for protein binding became apparent in recent years (*20*), challenging the traditional perception of a well-defined structure as a prerequisite for recognition and efficient interaction. The results of our work provide further indication that the two concepts are not necessarily contradictory and that disorder possibly evolves in complex biological systems alongside structure as a means of attaining the highest possible efficiency in biomolecular interactions.

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SUPPORTING INFORMATION AVAILABLE

Charge state deconvolution of the ionic signal of α-chains within the complex Hb mixture and isolated α-chains after reconstitution with a stoichiometric excess of the heme group at pH 8 and 4, ESI-MS of a mixture of α- and β-chains spiked with small amounts of a heme group, and the time course of α-globin reconstitution with a significant molar excess of a heme group. This material is available free of charge via the Internet at http://pubs.acs.org.

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