Assembly of Human Hemoglobin

STUDIES WITH ESCHERICHIA COLI-EXPRESSED α-GLOBIN*

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The α-globin of human hemoglobin was expressed in Escherichia coli and was refolded with heme in the presence and in the absence of native β-chains. The functional and structural properties of the expressed α-chains were assessed in the isolated state and after assembly into a functional hemoglobin tetramer. The recombinant and native hemoglobins were essentially identical on the basis of sensitivity to effectors (Cl⁻ and 2,3-diphosphoglycerate), Bohr effect, CO binding kinetics, dimer-tetramer association constants, circular dichroism spectra of the heme region, and nuclear magnetic resonance of the residues in the α₁β₁ and α₁β₂ interfaces. However, the nuclear magnetic resonance revealed subtle differences in the heme region of the expressed α-chain, and the recombinant human normal adult hemoglobin (HbA) exhibited a slightly decreased cooperativity relative to native HbA. These results indicate that subtle conformational changes in the heme pocket can alter hemoglobin cooperativity in the absence of modifications of quaternary interface contacts or protein dynamics. In addition to incorporation into a HbA tetramer, the α-globin refolds and incorporates heme in the absence of the partner β-chain. Although the CO binding kinetics of recombinant α-chains were the same as that of native α-chains, the ellipticity of the Soret circular dichroism spectrum was decreased and CO binding kinetics revealed an additional faster component. These results show that recombinant α-chain assumes alternating conformations in the absence of β-chain and indicate that the isolated α-chain exhibits a higher degree of conformational flexibility than the α-chain incorporated into the hemoglobin tetramer. These findings demonstrate the utility of the expressed α-globin as a tool for elucidating the role of this chain in hemoglobin structure-function relationships.

The basis of cooperative oxygen binding by human hemoglobin is an old problem that is being elucidated with the aid of modern technologies. Of particular importance is the elucidation of the properties of α- and β-chains, both in the isolated state and incorporated into the hemoglobin tetramer. Such studies should provide fine details on the linkage between tertiary and quaternary structure that forms the basis of cooperative oxygen binding. The recent development of hemoglobin expression systems offers great promise for facilitating the study of such structure-function relationships. This approach has been particularly useful for investigating the physicochemical properties of hemoglobin and myoglobin and has been employed in the development of hemoglobin variants with properties suitable for clinical use as red cell substitutes (1).

Although Escherichia coli expression systems for HbA have been developed, the product exhibits structural and functional modifications relative to native HbA (2–4). Alternatively, tetrameric hemoglobins in which only the α- or β-subunits are recombinant (αrHbA and βrHbA) can be obtained by in vitro refolding of the expressed globin. β-Globin has thus been expressed (5, 6) and refolded in the presence of native α-chains to yield recombinant hemoglobin whose conformational and functional properties resemble those of natural HbA (5, 6). In contrast, development of an analogous α-globin expression system was not as successful (7), although it is highly desirable as a tool for elucidating the tetrameric assembly and cooperativity of HbA.

In this paper we report the construction of a system for the synthesis of high levels of an α-globin fusion protein in E. coli. The conformational properties of the isolated chain are presented along with the functional and conformational characterization of reconstituted HbA tetramer following refolding of the expressed α-globin with heme in the presence of native β-chains. Spectral measurements and oxygen binding of these proteins reveal new insights into the linkage between the conformation of the heme pocket, ligand access channel, and quaternary interactions.

MATERIALS AND METHODS

Plasmid Construction—The α-globin expression plasmid pNFα is structurally analogous to pJKO5, from which β-globin has been routinely produced as a fusion protein, and comprises 83 residues of a flu virus protein, NS1, a Factor X recognition sequence followed by β-globin. The construction of pNFα is similar to that of pJKO5 (6). Human α-globin cDNA was provided by B. G. Forget (Yale University) as a 700-base pair PstI insert in pRT18 (8). In an initial effort to express α-globin in E. coli, the 700-base pair PstI fragment was inserted into the

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1 The abbreviations used are: HbA, human normal adult hemoglobin; αr, recombinant α-chains; βrHbA, human hemoglobin with recombinant β-chain; βrHbA, human hemoglobin with recombinant β-chain; Bis-Tris, [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; HPLC, high performance liquid chromatography; TPCK, 1-tosylamide-2-phenylethyl chloromethyl ketone; FXa, activated Factor X.
Expression and Purification of the α-Globin—Growth, expression, and purification of the fusion protein followed a previously described protocol (6). Enzymatic cleavage was monitored by reverse phase HPLC using a Vydac C4 column. The solvents were: A, 20% CH3CN, 0.1% trifluoroacetic acid; and B, 60% CH3CN, 0.1% trifluoroacetic acid. The gradient was 44% B to 75% B over 120 min. Peaks were identified by eight cycles of Edman sequencing.

Reconstitution of αHbA—Upon cleavage with Factor X, the α-globin was recovered as a precipitate, dissolved in a minimum volume of cold 0.1 M NaOH, and diluted to 2 mg/ml (A280 = 1.0 is taken as 1 mg/ml) with 0.04 M borate buffer, pH 9.0, 0.002 M EDTA. In order to solubilize the aggregates, the solution was deoxygénated by stirring under a constant stream of N2 followed by addition of dithiothreitol at a final concentration of 0.001 M. After 24 h at 4°C the protein was reconstituted with cyanoheme and native β-globin by first digesting pNa with NcoI and making the DNA blunt ended with mung bean nuclease. Subsequent digestion with BglII removed the NcoI portion of the fused gene. This segment was replaced by the BglII to Stul fragment from pJKO1 (6), which contains the same NcoI portion followed by a Factor X recognition sequence. The resulting plasmid, pNFα, is structurally identical to pJKO5 except that α-globin cDNA replaces the β-globin cDNA of pJKO5. This plasmid in strain AR120, induced with nalidixic acid, yielded large amounts of the NS1-FX-α-globin fusion protein as the principal component of the insoluble inclusion bodies.

NMR Measurements—The NMR experiments were carried out on a VXR-400/54 spectrometer operating at 9.4 tesla. All NMR spectra reported here were obtained at 29°C using the jump-and-return pulse sequence 90°(τ)−36°(τ)−90°(τ)−36°(τ) (20). The delay τ was adjusted for each experiment such that maximum excitation was obtained for the spectral region of interest. The relaxation delay between successive transients was 2.5 s. The proton chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (22).

The samples of native HbA and αHbA used for the NMR measurements were in 0.1 M Bis-Tris buffer, pH 6.85, in 90% H2O, 10% D2O. Hemoglobin concentration was between 7 and 9%, and methemoglobin content was 5% or less.

RESULTS

Protein Expression, Digestion, and Refolding—Fig. 1 shows SDS-polyacrylamide gel electrophoresis of the total cell proteins before and after induction as well as the detergent-insoluble fusion protein. This figure illustrates the high expression level of NS1-FX-α-globin and shows that treatment of the insoluble fraction with detergent eliminates a large part of the contaminant proteins.

The procedures for purification, cleavage, and reconstitution of the tetrameric hemoglobin were essentially the same as those used for βHbA (6). The pH values of the various reactions were increased due to the higher isolectric point of the hemoglobin.

2 In earlier NMR investigations of hemoglobin, proton chemical shifts have been referenced to the signal of water in each sample that is 4.73 ppm downfield from that of 2,2-dimethyl-2-silapentane-5-sulfonate at 29°C. For a detailed presentation of the conversion of the chemical shift scale in NMR spectra of hemoglobin, see Ho (22).
α-globin, which renders it less soluble than β-globin at pH 8.5. The yield of αHbA was 15–20 mg/liter of cell culture. Cleavage of the fusion protein with Factor Xa was followed by reverse phase HPLC. Fig. 2A shows the elution pattern of the detergent-purified fusion protein, and Fig. 2B shows the elution pattern of the fusion protein following 20 h of digestion with Factor Xa. The major peak corresponds to α-globin with the correct amino-terminal end, whereas the smaller peak is a product of overdigestion, which occurs at Argα31 (B12).

Peptide Maps of α-Chain from Normal Human HbA and rαHbA—Fig. 3, A and B, shows the tryptic peptide patterns obtained from normal and recombinant α-globins, respectively. The numbers above the peaks correspond to the tryptic fragments of the α-chain as listed in Table I. The HPLC profiles of tryptic digestion of the two α-globins are identical. Furthermore, five cycles of Edman sequential degradation on intact α-globins isolated from natural and recombinant HbA gave comparable yields of the expected 5 residues at the amino terminus. Sequence analysis of the peak eluting at 22 min identified an unresolved mixture of fragments 1 (residues 1–7) and 3 (residues 12–16). The peak eluting at 24 min contains fragment 1 + 2, a partial cleavage product (residues 1–11). The difference between peaks 12 and 13 eluting at 52 and 54 min is 1 Lys residue located at position α61 (E10).

Circular Dichroism—The spectrum in the Soret region is sensitive to the interaction of the heme with the surrounding aromatic residues (21). Fig. 4A shows the CD spectra of the carboxyl derivatives of HbA and αHbA. Although these exhibit the same ellipticity at the peaks of their spectra, small differences are evident at lower wavelengths. Fig. 4B shows the CD spectra of native and recombinant α-chains. The recombinant α-chains have a lower ellipticity and a broader spectrum than the native α-chains.

Nuclear Magnetic Resonance—Fig. 5 shows the downfield region of the NMR spectra of αHbA and HbA in the deoxy form. The resonances between 15 and 24 ppm originate from protons in the heme groups and/or from protons in amino acid residues located in the heme pockets. These resonances are shifted downfield by the hyperfine interactions between the corresponding protons and the unpaired electrons of the iron atoms. In deoxy-HbA, the hyperfine shifted resonances at 22.2 and 18.9 ppm have been assigned to the β-subunits, and those at 20.3 and 16.8 ppm have been assigned to the α-subunits (22–24). As shown in the figure, in deoxy-αHbA the same hyperfine shifted resonances are observed, and their chemical shifts are, within experimental error, the same as in deoxy-HbA. The spectral region from 11 to 15 ppm in Fig. 5 contains several other hyperfine shifted resonances and four exchangeable proton resonances. The hyperfine shifted resonances are significantly broader than the exchangeable proton resonances (i.e. 350–500 versus 50–75 Hz). Due to these differences in the line widths and to the spectral overlap, only the exchangeable proton resonances in the spectral region 11–15 ppm in Fig. 5 can be observed accurately. In deoxy-HbA, these four exchangeable proton resonances have been assigned to specific hydrogen bonds in the Hb molecule as follows: the resonance at 14.1 ppm to the hydrogen bond between Tyrα42(C7) and Aspβ99(G1) at the αββ interface (25, 26); the resonance at 13.0 ppm to the hydrogen bond between Aspα126(H9) and Tyrβ41(C1) at the αβ interface; the resonance at 12.2 ppm to the hydrogen bond between Hisα103(G10) and Asnβ108(G10) at the αββ interface (26); and the resonance at 11.1 ppm to the hydrogen bond between
Asp$^{94}$ (G1) and Trp$^{37}$ (C3) at the $\alpha_1\beta_2$ interface (27).

As shown in the figure, in deoxy-arHbA these four exchangeable proton resonances are very close if not identical to those in deoxy-HbA.

Our NMR results for arHbA in the ligated state are shown in Figs. 6 and 7. Fig. 6 shows the spectral region from 9.5–14.5 ppm. In carboxy-HbA, the resonances at 12.95, 12.1, and 10.2 ppm originate from exchangeable protons and have been assigned as follows: the resonance at 12.95 ppm to the hydrogen bond between Asp$^{126}$ (H9) and Tyr$^{35}$ (C1) at the $\alpha_1\beta_1$ interface (26); the resonance at 12.1 ppm to the hydrogen bond between His$^{103}$ (G10) and Asn$^{108}$ (G4) at the $\alpha_1\beta_1$ interface (26); and the resonance at 10.23 ppm to the hydrogen bond between Asp$^{94}$ (G1) and Asn$^{102}$ (G4) at the $\alpha_1\beta_2$ interface (25). In carboxy-arHbA, these exchangeable proton resonances occur at the same spectral positions as those in carboxy-HbA.

The spectra shown in Fig. 6 also contain several resonances from the heme groups. In carboxy-HbA these resonances are (28, 29): the resonance at 10.45 ppm (mesoproton $\gamma$ of the heme in $\alpha$-chains); the resonance at 10.1 ppm (mesoproton $\alpha$ of the
heme in the β-chain); and the resonance at 9.7 ppm (mesoproton δ of the hemes in α- and β-chains and mesoproton α of the heme in the α-chain). In carboxy-arHbA, the latter two heme resonances occur at the same positions as in carboxy-HbA. However, the resonance of the mesoproton γ of the heme in α-chains (10.45 ppm) is missing from the spectrum.

Fig. 7 shows the region from −2.2 to −0.4 ppm of the NMR spectra of carboxy-arHbA and HbA. This region contains resonances from protons in the heme groups and/or amino acids in the heme pockets that are shifted upfield by the ring-current effect of the heme groups. In carboxy-HbA, the resonance at −1.77 ppm has been assigned to the γ-CH₃ protons of the distal Val residues in the α- and β-subunits, Val₁₁₁(E11) and Val₁₁₁(E11) (28). As shown in Fig. 7, the position of this resonance in carboxy-arHbA is nearly identical to that in carboxy-HbA.

Functional Studies—The binding isotherms of HbA and arHbA were measured using 1.5–2.0 mM heme, a concentration at which dimers and their associated complications are negligible. For these experiments, heme oxidation was below 5%, thus eliminating the need for adding the reducing system. The curves were analyzed according to the Adair equation (17) and yielded the thermodynamic parameters listed in Table II. Although these parameters do not differ significantly within the 66.7% confidence limit, the cooperativity of arHbA is decreased at all oxygen fractional saturations as shown in Fig. 8.

The oxygen binding parameters of HbA and arHbA were measured in the presence of Cl⁻ and 2,3-diphosphoglycerate. The data (Table III) indicate that the oxygen affinity and cooperativity of natural HbA and arHbA are similarly affected by these effectors and that these exhibit the same Bohr effect.

CO Recombination Kinetics—The kinetics of CO binding to native HbA and arHbA were measured (Fig. 9) and the data analyzed using a multieponential model. Each binding curve was well represented by two components whose rates varied by about 30-fold (Table IV). The slower phase originated from CO binding to the tetramer, and the fast phase originated from CO binding to the dimer as described previously (30). Comparison of the rate constants shows that HbA and arHbA exhibit similar rates of CO recombination for both dimers and tetramers.

The kinetic analysis also yields absorbance values that reflect the amounts of tetramer and dimer. These values were used to calculate the dimer-tetramer association constant (Kₐ) and the accompanying ΔG value for this transition (Table IV). The resulting Kₐ values for HbA (2.93 × 10⁶ M⁻¹) and arHbA (2.0 × 10⁶ M⁻¹) and their respective ΔG values (~7.34 and ~7.12 kcal/mol) were similar.

The kinetics of CO binding to native and recombinant α-chains were also measured. Each binding curve was well represented by two components whose parameters are presented in Table V. The major components of the native and recombinant α-chains reacted at the same rate (kₐ = 18.2 and 19.7 × 10⁵ M⁻¹ s⁻¹, respectively). However, the two samples differed significantly in that the major component corresponded to virtually the entire (97%) native α-chain but to only 63% of the recombinant α-chain.
DISCUSSION

The long term objective of this work is elucidation of the role of α-chains on the structure and function of hemoglobin. Our strategy involved comparison of the expressed and native α-chains both as the isolated chain or incorporated into the HbA tetramer. Any observed differences in conformational or functional properties can then be attributed to the α-chain. Thus, although the αrHbA was almost identical to native HbA, the subtle differences that were observed provide new details on the role of proper α-chain folding in maintaining the structure and cooperativity of the hemoglobin molecule. The conformational and functional aspects will be considered separately.

FIG. 6. Exchangeable and heme proton resonances of carboxy-HbA (lower spectrum) and carboxy-αrHbA (upper spectrum) in 0.1 M Bis-Tris buffer, pH 6.85, in 90% H₂O, 10% D₂O at 29°C.

FIG. 7. Ring-current shifted proton resonances of carbonmonoxy HbA (lower spectrum) and carbonmonoxy αrHbA (upper spectrum) in 0.1 M Bis-Tris buffer, pH 6.85, in 90% H₂O, 10% D₂O at 29°C.
Table II
Comparison of the oxygen binding parameters recovered for HbA and arHbA

| Buffer, 50 mM HEPES, pH 7.4; temperature, 25 °C. | HbA | arHbA |
|-----------------------------------------------|-----|-------|
| $K_a \times 10^5$ (mol$^{-1}$)                 | $1.5 \pm 0.3$ | $1.9 \pm 0.3$ |
| $K_a \times 10^5$ (mol$^{-1}$)                 | $0.9 \pm 0.3$ | $1.3 \pm 0.2$ |
| $K_a \times 10^5$ (mol$^{-1}$)                 | $2.5 \pm 1.5$ | $0.8 \pm 0.4$ |
| $K_a \times 10^5$ (mol$^{-1}$)                 | $33.0 \pm 7.0$ | $31.0 \pm 6.0$ |
| $\Delta G^i$ (kcal/mol)                       | $-1.8 \pm 0.3$ | $-1.6 \pm 0.2$ |
| $P_m^a$ (torr$^{-1}$)                          | $2.0 \pm 0.3$ | $2.4 \pm 0.3$ |
| $n_{max}^a$                                    | 2.3   | 2.0    |

$^a$ Cooperative free energy = $-RT \ln K_a/K_1$.

$^b$ Median ligand activity.

$^c$ Hill parameter.

Fig. 8. Cooperative activity of natural HbA (solid line) and recombinant HbA (dashed line) at increasing fractional saturation with oxygen. Measurements were done in 50 mM HEPES buffer, pH 7.4, at 25 °C.

Table III
Oxygen equilibrium data were obtained using the thin layer dilution method of Dolman and Gill

| Buffer, 50 mM HEPES, pH 7.4; protein concentration, 30 mg/ml; and temperature, 25 °C. | Pm | n_{max} | Bohr effect ($\Delta H/\Delta pH$) |
|-------------------------------------------------------------------------------------|----|---------|----------------------------------|
| HbA (no NaCl)                                                                       | 2.0 ± 0.3 | 2.3 |
| HbA (0.2 M NaCl)                                                                    | 4.51 ± 0.01 | 3.0 |
| $\triangle \log P_m \pm NaCl$                                                        | 0.35 |
| HbA (0.01 M DPG)                                                                    | 10.0 ± 0.1 | 3.1 |
| $\triangle \log P_m \pm DPG$                                                        | 0.7 |
| HbA (0.1 M NaCl)                                                                    | 0.65 (pH = 7.4) |
| arHbA (no NaCl)                                                                     | 2.4 ± 0.3 | 2.0 |
| arHbA (0.2 M NaCl)                                                                  | 5.85 ± 0.01 | 2.6 |
| $\triangle \log P_m \pm NaCl$                                                        | 0.39 |
| arHbA (0.01 M DPG)                                                                  | 12.3 ± 0.1 | 2.6 |
| $\triangle \log P_m \pm DPG$                                                        | 0.7 |
| arHbA (0.1 M NaCl)                                                                  | 0.65 (pH = 7.4) |

$^a$ DPG, 2,3-diphosphoglycerate.

Conformational Studies—The NMR spectra in Fig. 5 indicate that the overall conformation of the heme pockets in deoxy-arHbA is similar to that in deoxy-HbA. A subtle difference, however, is observed for the relative intensities of the hyperfine shifted resonances at 22.2 and 16.8 ppm in arHbA. In deoxy-HbA, the intensity of the $\alpha$-chain hyperfine shifted resonance at 16.8 ppm is the same as that of the $\beta$-chain hyperfine shifted resonance at 22.2 ppm (namely, six protons/heme) (31). In contrast, in deoxy-arHbA the $\alpha$-chain resonance at 16.8 ppm appears more intense than the $\beta$-chain resonance at 22.2 ppm.

The NMR spectra shown in Fig. 5 have been obtained using the jump-and-return pulse sequence with maximum excitation at the $\alpha$-chain hyperfine shifted resonance at 16.8 ppm. Due to the frequency-dependent excitation profile of the pulse sequence used, the intensity of the $\beta$-chain hyperfine shifted resonance at 22.2 ppm shown in the figure corresponds to 75% of its actual value.

In order to understand the origin of this difference we have measured the absolute intensity of the hyperfine shifted resonance of arHbA using a reference standard (tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionate) europium) complexed with t-butyl alcohol (31). The results showed that the $\beta$-chain resonance at 22.2 ppm in arHbA has the same intensity as the corresponding resonance in HbA. In contrast, the intensity of the $\alpha$-chain resonance at 16.8 ppm in arHbA is increased by a factor of approximately 2 relative to that in HbA. The origin of this difference is difficult to ascertain since, at present, it is not known whether the $\alpha$-chain hyperfine shifted resonance at 16.8 ppm originates from protons in the heme groups, from protons in the amino acids in the heme pockets, or from both. Future NMR experiments on HbA molecules containing fully deuterated heme groups may provide an answer to this question.

Similarly, in carboxy-arHbA (Fig. 6) the resonance of the mesopron $\gamma$ of the heme in the $\alpha$-chain (10.45 ppm) is missing from the spectrum, probably shifted to a different spectral position in which it cannot be resolved. This result suggests that in the ligated form the heme environment in the $\alpha$-subunits of arHbA may also differ from that in HbA. Alterations in the heme packets are also suggested by the ring-current shifted resonances (Fig. 7). All these resonances are broader in arHbA than in native HbA, and in arHbA the resonance at $\sim$0.64 ppm appears to be shifted from its position in HbA. Similar differences in the ring-current shifted resonances have been previously observed for a full recombinant HbA (4) and have been attributed to a different mode of heme insertion. One possibility previously suggested is the existence of two heme orientations that differ by a 180° rotation about the $\alpha$, $\gamma$-mesoaxis of the porphyrin (32). Our results for arHbA do not support this suggestion, since a rotation about the $\alpha$, $\gamma$-mesoaxis would conserve the environment of the $\gamma$-mesopron. In contrast, our results show that in arHbA the $\gamma$-mesopron resonance is shifted from its position in the spectrum of HbA (Fig. 6). Moreover, our results show that the position of the distal (E11)Val residues relative to the heme groups in ligated arHbA is very close to that in HbA. This is consistent with the observed similarity of the CD spectra (Fig. 4A) and indicates that the small difference observed near 410 nm is not due to heme inversion. Further investigations are necessary in order to characterize fully the conformation of the $\alpha$-chain heme pockets in arHbA. Figs. 5 and 6 show that the exchangeable proton resonances of deoxy-arHbA are very similar if not identical to those in HbA in both deoxy and carboxyl forms. These findings...
The free energy of cooperativity, $D_G$, is the dimer-tetramer assembly free energy calculated from the dimer-tetramer association equilibrium constant. Conditions: 0.1 M Bis-Tris and 0.1 M KCl, pH 7.0, 23°C.

### Table IV

Kinetic parameters, dimer-tetramer equilibrium constants, and dimer-tetramer assembly free energies for HbA and arHbA

$k_1$ and $k_2$ are the bimolecular association rate constants for CO binding to tetramer and dimer, respectively, and $a_1$ and $a_2$ are the absorbance parameters for tetramer and dimer, respectively. $K_a$ is the dimer-tetramer association equilibrium constant calculated from the $a_1$ and $a_2$ values. $\Delta G$ is the dimer-tetramer assembly free energy calculated from the dimer-tetramer association equilibrium constant. Conditions: 0.1 M Bis-Tris and 0.1 M KCl, pH 7.0, 23°C.

|       | $a_1$    | $k_1$ ($\times 10^{-5} \text{ M}^{-1} \text{s}^{-1}$) | $a_2$    | $k_2$ ($\times 10^{-5} \text{ M}^{-1} \text{s}^{-1}$) | $K_a$ ($\times 10^{-5} \text{ M}^{-1}$) | $\Delta G$ (kcal/mol) |
|-------|---------|------------------|---------|------------------|------------------|------------------|
| HbA   | 0.081 ± 0.014 | 0.62 ± 0.14     | 0.104 ± 0.016 | 20.5 ± 3.8     | 2.93 ± 1.02     | −7.34 ± 0.23    |
| arHbA | 0.063 ± 0.019 | 0.73 ± 0.13     | 0.105 ± 0.023 | 19.0 ± 2.5     | 2.00 ± 0.63     | −7.12 ± 0.22    |

### Table V

Kinetic parameters for native and recombinant $\alpha$-chains

$k_1$ and $k_2$ are the bimolecular association rate constants for CO binding to the two components of native and recombinant $\alpha$-chains, and $a_1$ and $a_2$ are the corresponding absorbance parameters. Conditions: 0.1 M Bis-Tris and 0.1 M KCl, pH 7.0, 23°C.

|       | $a_1$ ($\times 10^{-5} \text{ M}^{-1}$) | $k_1$ ($\times 10^{-5} \text{ M}^{-1} \text{s}^{-1}$) | $a_2$ ($\times 10^{-5} \text{ M}^{-1}$) | $k_2$ ($\times 10^{-5} \text{ M}^{-1} \text{s}^{-1}$) |
|-------|------------------|------------------|---------|------------------|
| Native $\alpha$ chain | 0.005 ± 0.001 | 2.35 ± 0.77     | 0.165 ± 0.005 | 18.2 ± 1.6     |
| ar chain | 0.062 ± 0.014 | 8.21 ± 0.83     | 0.107 ± 0.010 | 19.7 ± 1.4     |

The results suggest that substitution of the recombinant for the native $\alpha$-globin is sufficient to alter these oxygenation intermediates independently of the quaternary assembly and propose a key role for nonquaternary interactions in the modulation of oxygen cooperativity.

Functional Studies—The thermodynamic analysis reported in Table II shows that HbA and arHbA exhibit oxygen binding properties that are indistinguishable except for a reduced cooperativity. Since the NMR data indicate identical resonances for residues at the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ interfaces, the reduced cooperativity cannot be attributed to altered quaternary assembly. The free energy of cooperativity, $D_G$, is the same for HbA and arHbA ($−1.8 ± 0.3$ and $−1.6 ± 0.2$ kcal/heme). Thus, the decreased value of $n_{\text{max}}$ measured for arHbA is probably due to phenomena linked to the intermediate stages of oxygenation. At present the molecular events associated with the intermediates of oxygenation are still elusive, as these partially oxygenated forms are very unstable and their fractional distribution is much smaller than anticipated by a statistical distribution of ligands on tetrameric hemoglobin (34). These results suggest that substitution of the recombinant for the native $\alpha$-globin is sufficient to alter these oxygenation intermediates independently of the quaternary assembly and propose a key role for nonquaternary interactions in the modulation of oxygen cooperativity.

The Bohr effect and the oxygen affinity regulation by allosteric effectors are sensitive probes of tertiary structure and quaternary assembly. In arHbA the Bohr effect and the sensitivity to Cl$^−$ and 2,3-diphosphoglycerate are equivalent to that in native HbA (Table III). This indicates that the Bohr effect groups and the effector binding sites are correctly aligned in the half-recombinant protein. This is probably favored in the half-recombinant hemoglobin by the native partner chains, which help direct the correct refolding and reassembly of the recombinant chains (35).

The CO binding experiments confirm the oxygen equilibrium data (Fig. 9 and Table IV) and reveal that HbA and arHbA are indistinguishable on the basis of two additional criteria: rate of CO binding and the energetics of dimer-tetramer association. Our observation that the major component of both recombinant and native $\alpha$-chains exhibited the same rate (19.7 and 18.2 × 10$^{-5}$ M$^{-1}$ s$^{-1}$) as that of the dimers of native HbA (20.5 × 10$^{-5}$ M$^{-1}$ s$^{-1}$) and arHbA (19.5 × 10$^{-5}$ M$^{-1}$ s$^{-1}$) is consistent with a similar conformation of isolated $\alpha$-chains, whether monomeric or polymeric, and the $\alpha$-chains within a hemoglobin dimer. The differences observed between the binding kinetics of the expressed and native $\alpha$-chains thus are eliminated during tetramer formation, as the energetics of quaternary assembly more than compensate for the decreased conformational flexibility of $\alpha$ upon its incorporation into the tetramer.

In addition to characterizing HbA, it is also important to determine the properties of the isolated chains. The ability of isolated $\alpha$-globin to refold with heme may perhaps be explained by the results from the CO binding kinetics and CD. The greater heterogeneity in CO binding kinetics of the $\alpha$-chain suggests a correspondingly greater degree of structural or dynamic heterogeneity relative to that of the native $\alpha$-chain. This result is consistent with the CD measurements, which reflect the time-averaged structure of all $\alpha$-chain components and which show reduced ellipticity and a broader spectrum in the Soret region for the $\alpha$-chains. Taken together, these approaches suggest the presence of alternating conformations for the isolated $\alpha$-chain. A dynamic access to a wide conformational space would enhance its prospects for correctly refolding with the heme and ultimately attaining a conformation suitable for pairing with $\beta$-chain and formation of a functional HbA tetramer.

### CONCLUSION

In conclusion, we have expressed $\alpha$-globin that can refold with heme in the absence of the $\beta$-chain. The isolated $\alpha$-globin exhibits a wide range of conformational flexibility. Furthermore, this $\alpha$-globin can recombine with the heme and assemble...
with β-chain to produce a recombinant HbA whose functional and conformational characteristics resemble those of natural HbA. Although subtle modifications in the heme pocket and a decreased cooperativity are observed, the contacts across the α₁β₁ and α₁β₂ interfaces are retained. These findings underscore the key role played by the tertiary structure of the heme region, exclusive of the quaternary structure, in modulating the functionality of the HbA molecule. Our results also suggest a key role for nonquaternary interactions in processes involving the relatively unstable oxygenation intermediates. The expressed α-globin is thus suitable for construction of mutants to be used in future studies of HbA structure-function relationships.

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