Structural and Functional Properties of Human Hemoglobins Reassembled after Synthesis in *Escherichia coli*[†,‡]

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ABSTRACT: Human hemoglobin produced in the *Escherichia coli* coexpression system of Hernan et al. [(1992) *Biochemistry 31,* 8619−8628] has been transformed into a functionally homogeneous protein whose properties closely approximate those of normal hemoglobin A. Both of the α and *β* chains of this hemoglobin contain a valine−methionine substitution at position 1 in order to accommodate the difference in specificity of the protein-processing enzymes of procaryotes. Despite extensive purification, functional homogeneity of the *E. coli* expressed hemoglobin was achieved only by the complete disassembly of the hemoglobin into its component α and *β* globins and their reassembly in the presence of hemin. The kinetics of CO combination and the thermodynamics of O_2 binding and cooperativity of the reassembled αV1M−*β*V1M hemoglobin closely approximate those of HbA. The α globin obtained from the *E. coli* expressed hemoglobin was also combined with normal human *β* chains and hemin to form the αV1M variant. The α+M variant of HbA, in which the normal N-terminal valine of the α chains is preceded by a methionine residue, was prepared by the same procedure. The kinetics of the reactions of CO with the αV1M and α+M variants are similar to those for HbA. The equilibria of oxygen binding to αV1M and HbA are similar whereas α+M exhibits a significantly higher oxygen affinity. The three-dimensional structures of αV1M and α+M offer an explanation for the latter affinity difference. Although the structures of αV1M and HbA, which have been determined by X-ray crystallography, are virtually indistinguishable except at the N-terminal residues, that of α+M indicates the displacement of a solvent molecule, possibly a chloride ion, from arginine 141α. Such an alteration in an anion binding site could result in increased oxygen affinity.

Allosteric regulation permeates the living world, providing mechanisms for many important functions, including the control of enzyme activities, communication across cell membranes, regulation of gene transcription, and intracellular transport, among others. Continuing interest in the study of human hemoglobin is thus motivated both by this molecule's crucial physiological importance and by its role as a prototype for understanding allosteric mechanisms. Although our knowledge of hemoglobin far exceeds that of any other protein which displays complex functional properties, the mechanistic details of cooperative oxygen binding are still only partially known. Studies of the relationships between the structural and functional properties of hemoglobin have been greatly facilitated by the identification of many naturally occurring mutations of the human protein. However, the use of these naturally occurring mutants has a number of shortcomings. Although the total list of human hemoglobin mutations is impressive, it is remarkable that not even the majority of these mutants are generally available to investigators nor is their supply reliable. Thus, only a fraction of the known mutants has been examined in detail, and some are very difficult to separate from HbA because of identity in their charges. Most significantly, the naturally occurring mutations rarely represent the amino acid substitution that one would choose for the study of a particular question. For example, if one wishes to examine the functional importance of the normal residue at a particular sequence position, the ideal substitution may be an alanine or glycine residue which essentially removes the native Hb side chain and replaces it with the smallest possible structures, such as a methyl group or a hydrogen.

Modern advances in molecular genetics have permitted the expression of hemoglobin in microorganisms and the precise modification of the structure of the protein by means of site-directed mutagenesis. In *E. coli*, individual globin proteins can be expressed as either fusion proteins (*1*−*3*) or, in the case of *β* globin, the apoprotein itself (*4*). It is

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[‡] Refined coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank. The accession numbers for the coordinates and the structure factors are 1bz0 and r1bz0sf, 1bz1 and r1bz1sf, and 1bzz and r1bzzsf for HbA, α+M, and αV1M, respectively.

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possible to coexpress α and β globins in *E. coli* strains which respond by increasing heme production with the resulting production of intact, heme-containing molecules (*4*−*6*). The coexpression system described by Hernan et al. (*4*) is built around synthetic α and β globin genes designed for optimal codon usage in *E. coli*. These are positioned downstream from a *lac* promoter. In 1995, Hernan and Sligar (*7*) reported the purification and the characterization of the physical and functional properties of the hemoglobin formed in this system. Because of the specificities of the bacterial processing enzymes, the initiator methionine is not cleaved from the nascent globin chain. Therefore, the NH_2-terminal valine codon was deleted from both genes, resulting in a Val → Met substitution at the first sequence position of both globins. Although the hemoglobin obtained was pure as judged by HPLC[1] and mass spectroscopy, CO combination kinetics revealed extreme functional heterogeneity. Suspecting incorrect assembly of the hemoglobin molecules within the bacteria, these authors separated the α and β chains of the recombinant hemoglobin and then mixed them together to permit recombination. This procedure greatly reduced the kinetic heterogeneity and yielded properties closer to those of normal human HbA. However, significant functional heterogeneity persisted.

We now report a procedure by which the hemoglobin produced in the coexpression system of Hernan et al. (*4*) can be transformed into a functionally homogeneous protein whose properties closely approximate those of HbA. This involves the removal of heme to form the apoprotein, the separation of α and β globins, and their subsequent reassembly in the presence of cyanohemin to reform the intact tetrameric protein. Hernan et al. (*4*) examined the functional consequences of alternative NH_2-terminal structures on the β chain using their β globin expression system and concluded that the βV1M substitution best approximated the properties of native human HbA. A similar examination of alternative N-terminal structures of the α chain had never been carried out. Therefore, we have expressed hemoglobins with the αV1M substitution and with an NH_2-terminal Met in addition to the normal Val residue on the α chain, α+M. Hemoglobins can be reassembled by mixing either α and β globins with hemin or the globin of one type of subunit with its heme-containing partner chain and hemin. However, the yield is significantly greater if the latter procedure is used, and because we most frequently prepare hemoglobins bearing a mutation in only one type of chain, the latter technique is best suited. This procedure is routinely used to assemble hemoglobins from β globins obtained with the β globin expression system of Hernan et al. (*4*; see also ref *8*). In this study, we have prepared three reassembled hemoglobins: αV1M−βV1M hemoglobin, prepared from *E. coli* coexpressed α and β globins; αV1M hemoglobin, prepared from αV1M globin and normal human β chains; and α+M hemoglobin, prepared from normal human β chains and α globin containing the normal Val at position 1 preceded by the extra Met residue. The functional properties of these hemoglobins have been characterized, and the three-dimensional structures of deoxy αV1M and deoxy α+M have been determined by X-ray crystallography.

EXPERIMENTAL PROCEDURES

Normal Human HbA and β Chains from HbA. HbA was purified from hemolysates of freshly drawn blood by the method of Geraci et al. (*9*) as described by Doyle et al. (*10*). The β chains of HbA were obtained by the Geraci et al. (*9*) modification of the method of Bucci and Fronticelli (*11*).

Mutant Hemoglobins. These were expressed in *E. coli* strain TB-1 as described by Hernan et al. (*4*) using an operon composed of synthetic α and β globin genes downstream from a *lac* promoter. *E. coli* were grown in broth, containing 200 μg/mL ampicillin, to an absorbance of 0.3 at 600 nm. Addition of isopropyl β-D-thiogalactopyranose (IPTG) to a concentration of 1 mM induced the synthesis of α and β globins with the concomitant overproduction of heme and assembly of the holoprotein. Bacteria were harvested by centrifugation. Frozen cells (150 g) were thawed and suspended in 300 mL of a buffer containing 15 mM HCl/Tris (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 0.1 mM $MnCl_2$, 40 mg of RNase A (Sigma), 5 mg of RNase B (Sigma), and 10 000 units of DNase (Sigma). Cells were lysed by sonication in 100 mL batches using the Branson sonifier model 450 at an output of 230 W for 60 s. The sample was kept cold in an ethanol−ice bath. The broken cells were centrifuged for 20 min at 17 300*g* in a Sorvall SS-34 rotor. The supernatant was further clarified in the Beckman ultracentrifuge at 50 000 rpm for 30 min using the 55.1 Ti rotor at 5 °C. This supernatant was passed through a G25 coarse column (4 × 70 cm) preequilibrated in 15 mM HCl/Tris (pH 8.0) containing 0.1 mM EDTA and 1 mM DTT. The eluate was loaded onto a DE52 column (4 × 8 cm) equilibrated with the same pH 8.0 buffer. This column was then washed with 500 mL of the starting buffer to remove impurities which do not bind to the column. The hemoglobin fraction was eluted with a gradient of 500 mL each of the pH 8 buffer and 15 mM HCl/bisTris (pH 6.2) containing 0.1 mM EDTA and 1 mM DTT. All buffers were equilibrated with CO. The hemoglobin fraction was concentrated through PM30 Amicon membranes and stored frozen in liquid nitrogen.

HPLC Purification of E. coli Expressed Hemoglobin. HPLC fractionation was carried out with a Waters 650 preparative HPLC apparatus using two different columns, a DEAE 5PW TSK gel column and a SP 5PW TSK gel column (both Toso Haas, 2 × 15 cm). For fractionation on the DEAE column, the concentrated *E. coli* expressed hemoglobin fraction from the DE52 column was passed through a G25 course Sephadex column equilibrated with 15 mM HCl/Tris (pH 8). The sample was then loaded onto the DEAE 5PW TSK gel column which had been equilibrated with the same pH 8 buffer. The hemoglobin was eluted for 85 min with a gradient of from 15 mM HCl/Tris (pH 8) to 15 mM HCl/bisTris (pH 7) at a flow rate of 5 mL/min. For purification on the SP column, the G25 course column and the SP 5PW TSK gel column were equilibrated with 20 mM HCl/bisTris

[1] Abbreviations: bisTris, bis(2 hydroxyethyl)iminotris(hydroxymethyl)-methane; Tris, tris(hydroxymethyl)aminomethane; DE52, diethylaminoethyl cellulose anion exchange medium (Whatman); IHP, inositol hexaphosphate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; HbA, human hemoglobin A; αV1M, human hemoglobin variant with the valine at position 1 of the α chain replaced by methionine; α+M human hemoglobin variant with an additional methionine residue preceding the valine at position α1; αV1M−βV1M, human hemoglobin variant with both of the α1 and β1 valines replaced by methionine residues.

(pH 7). The hemoglobin was eluted from the SP column for 85 min with a gradient from 20 mM HCl/bisTris (pH 7) to 25 mM HCl/Tris (pH 8) at a flow rate of 5 mL/min.

Mutant α and β Globins. The *E. coli* expressed hemoglobin isolated by DE52 chromatography was used without further purification for preparation of α and β globins. Acetone was made acidic by adding 2.5 mL of 2 M HCl per liter of spectral HPLC grade acetone. Aliquots (5 mL) of hemoglobin at a concentration of approximately 0.5 mM in heme equivalents were added to 200 mL of acid acetone at −20 °C, followed by centrifugation at 7000 rpm in the Sorvall SS34 rotor for 10 min, at −20 °C. The globin pellet was dissolve in 8 M urea, 5 mM phosphate, and 50 mM mercaptoethanol (pH 6.7). The pH of this solution had to be adjusted with a few crystals of Tris base to pH 6.7. The sample was dialyzed overnight in the pH 6.7 urea buffer. Before being loaded onto a CM Sepharose CL6B column (2 × 7 cm) preequilibrated in the pH 6.7 buffer described above, the globin solution was spun at 50 000 rpm for 30 min in the Beckman 55.1 Ti rotor at 15 °C. After the sample was applied, the column was washed with approximately 80 mL of the pH 6.7 urea buffer until the nonbinding material was eluted. The β globin was eluted with a gradient of 180 mL each of pH 6.7 urea buffer and 0.1 N NaCl in the same urea buffer. The α globin was eluted with a second gradient of 0.1−0.2 M NaCl in the urea buffer. The eluted globin fractions were concentrated through PM10 Amicon membranes and stored frozen at −20 °C.

Reassembled E. coli Expressed Hemoglobin. The α and β globins from the *E. coli* expressed hemoglobin were combined and reacted with cyanohemin. Starting concentrations of α and β globins in the 8 M urea buffer were between 4 and 5 mg/mL. Molar extinction coefficients of 1.0×10^4 and 1.54×10^4 were used for α and β globins, respectively (*12*). Cyanohemin was in 1.2-fold molar excess of the combined globin concentrations. The globins were diluted to a final concentration of 0.3 mg/mL in cold deionized water which was equilibrated with CO. The final pH of the assembly mixture was adjusted to 8.0 with a few crystals of Tris base. The reaction mixture was left undisturbed overnight at 5 °C. The sample was concentrated through PM30 membranes on the following day. The concentrated reaction mixture, containing the reassembled hemoglobin, was reduced anaerobically with dithionite in the presence of CO and passed through a G25 coarse column (4 × 50 cm) to remove excess hemin and dithionite using a 15 mM HCl/Tris (pH 8.4) buffer. Separation of hemoglobin from α or β chains or both was accomplished on a DE52 column (4 × 5 cm) in 15 mM HCl/Tris (pH 8.4) buffer. After it was washed with 100 mL of the pH 8.4 starting buffer, a gradient of 750 mL each of starting buffer and 80 mM HCl/Tris (pH 8.0) buffer was applied. Excess α chains were eluted first from the column. Hemoglobin then eluted as the major band. The β chains do not migrate on this column with this gradient.

Mutant Hemoglobins Containing Only Mutant α Chains. Hemoglobins also were assembled from α globin produced in the coexpression system, cyanohemin, and normal human β chains, using essentially the same procedure described by Hernan et al. (*4*) for the assembly of hemoglobins from mutant β globin expressed in *E. coli*, cyanohemin, and normal α chains. The purified α globin in 8 M urea buffer was diluted into cold water, and a 1.2-fold excess of cyanohemin and β chains was added. After an overnight incubation, the sample was concentrated and purified as described for the reassembled *E. coli* expressed hemoglobin.

Kinetics of CO Combination with Deoxygenated Hemoglobin by Stopped Flow. These kinetics were measured with an OLIS U.S.A. (On Line Instrument Systems Inc., Bogart, GA) stopped-flow apparatus. The procedures were essentially those of Gibson (*13*) as described by Doyle et al. (*10*). Reactions were followed at 420 and 435 nm using a 1.7 cm path length cell. Concentrations of CO and hemoglobin (in heme equivalents) were 20 and 2 μM, respectively.

Kinetics of CO Recombination following Complete Photodissociation. Measurements were carried out as previously described (*10*). The three photographic strobe units were adjusted to give a rectangular light pulse approximately 0.5 ms in duration. The recombination reaction was followed at 420 and 435 nm. Hemoglobin and CO concentrations were the same as those in the measurements of CO combination by rapid mixing.

Both types of kinetic measurements were carried out in 100 mM HCl/bisTris buffers at pH 7 and below and in 100 mM HCl/Tris buffers above pH 7. Dithionite was present at a concentration of approximately 2 mM in order to maintain anaerobic conditions and to keep the heme iron atoms in their ferrous state. Data acquisition and processing were carried out by an OLIS model 4000 data acquisition and instrument control system. Kinetic data were fitted to single or double exponential functions using successive integration or Levenberg−Marquardt fitting routines (*8*).

Thermodynamics of and Linkage between Oxygen Binding and Dimer−Tetramer Assembly. Deoxygenated assembly equilibria for the formation of tetramers from dimers were measured kinetically as described by Doyle et al. (*10*). The rate constant for dissociation of the tetramer into two αβ dimers was measured by the haptoglobin technique of Ip et al. (*14*). The equilibrium constant for the deoxygenated tetramer's assembly, ${}^0K_2$, was calculated from the measured dissociation rate constant along with an association rate constant of 1.1 (±0.2) × 10^6 M^{-1} s^{-1}. The latter is a consensus value which has been found to be invariant over wide ranges of pH, temperature, and mutagenic perturbations (*15*).

Oxygen-binding isotherms were measured as described by Doyle et al. (*10*), using the continuous-flow spectrophotometric method of Imai (*16*) as described by Chu et al. (*17*). All oxygenation and haptoglobin kinetic measurements were performed in 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA at 21.5 °C and pH 7.4 (0.18 M total chloride).

The linkage between oxygenation of the hemoglobin system and dimer−tetramer assembly was analyzed according to the linkage diagram of Figure 1 (*10*). Free-energy terms are adjacent to arrows which represent equilibria. The dashed arrow at the doubly ligated tetrameric state indicates that there are two ligand stoichiometry combinations of dimers which contribute to its formation. Thermodynamic parameters of the linkage diagram were determined by global regression analysis of oxygenation isotherms as described by Doyle et al. (*10*) and Kiger et al. (*18*).

X-ray Crystallography of Deoxy αV1M and α+M. Purified α+M and αV1M were dialyzed into 10 mM ammonium phosphate (pH 7.0) prior to crystallization. Crystals of the mutant deoxyhemoglobins, grown according to the procedure

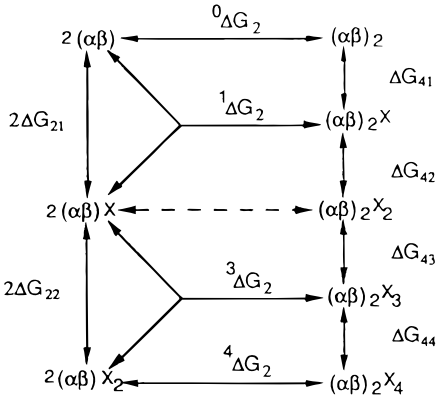

FIGURE 1: Linkage diagram showing stepwise O_2 binding free energies, ΔG_{4i}, of tetrameric hemoglobin species with *i* bound oxygens and their thermodynamic linkages to the corresponding dimer−tetramer assembly reactions (having free energies $^i\Delta G_2$). O_2 is denoted by X. The dissociated dimers have stepwise binding free energies denoted by ΔG_{21} and ΔG_{22}. Experimental measurements of the extent of O_2 saturation, *Y*, at a series of hemoglobin concentrations may be analyzed by nonlinear regression to yield these free-energy parameters that reflect the affinities and cooperativities of the hemoglobin species depicted.

of Perutz (*19*), were isomorphous with deoxy HbA crystals (space group $P2_1$ with $a = 63.3$ Å, $b = 83.6$ Å, $c = 53.8$ Å, and $\beta = 99.4°$). Diffraction data were collected on a Rigaku AFC6 diffractometer fitted with a San Diego Multiwire Systems area detector. A single crystal of each mutant was used for data collection. A total of 399 325 measurements were made of 61 802 unique reflections for the α+M mutant, and 448 711 measurements were made of 64 636 unique reflections for the αV1M mutant. The diffraction data were scaled and merged according to the method described by Howard et al. (*20*). The data for deoxy α+M are 83.9% complete out to 1.6 Å resolution and 66.6% complete for the highest resolution shell (1.72−1.59 Å). Similarly, the αV1M diffraction data are 88.7% complete out to 1.6 Å resolution and 73.4% complete for the highest resolution shell. The 1.6 Å data are of good quality, as indicated by R_{symm} values of 5.3% and 4.8% on intensity for the α+M and αV1M mutants, respectively. The diffraction data were randomly split into a working set (90% of the data) for refinement and a test set (10% of the data) for R_{free} cross validation (*21*).

The refinement protocol of the deoxy α+M and αV1M crystal structures consisted of rigid-body refinement using X-PLOR (*22*) followed by restrained least-squares refinement with PROLSQ (*23*, *24*). The 1.5 Å structure of deoxy HbA (*25*) was used as the initial atomic model for both refinements, except that Val1α was converted to an alanine in the case of the αV1M refinement. The standard crystallographic *R* values for the initial α+M and αV1M models were 0.188 and 0.178, respectively, for the data between 8.0 and 1.6 Å resolution with magnitudes greater than 2σ (54 174 reflections for α+M and 57 541 reflections for αV1M). Twenty cycles of rigid-body refinement of the entire tetramer, followed by 20 refinement cycles of the two αβ dimers, and finally 20 refinement cycles of the four individual subunits resulted in an *R* value of 0.183 for α+M and 0.172 for αV1M. Following rigid-body refinement, the α+M and αV1M structures were subjected to 15 cycles of restrained least-squares refinement. The NH_2-terminal methionine was then added to the α+M model, and the first residue of the

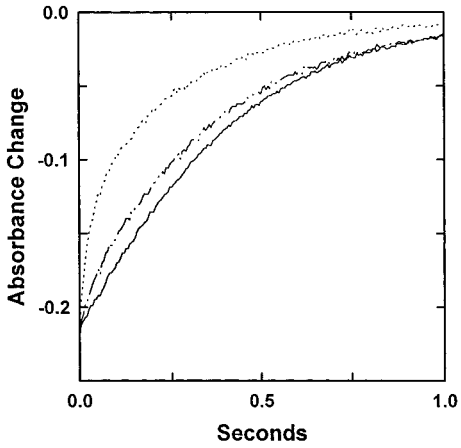

FIGURE 2: Time courses for the combination of CO with HbA (−), *E. coli* expressed hemoglobin purified by DEAE cellulose chromatography (···), and *E. coli* expressed hemoglobin purified by HPLC (-··-). Reactions were carried out at 20 °C in 0.1 M HCl/bisTris buffer (pH 7).

αV1M model, Ala1α, was converted to a methionine. The methionine residues were fit into electron-density images using the TOM/FRODO software (*26*, *27*). Electron-density images were calculated as $|F_{obs} - F_{calc}|$ Fourier maps in which the NH_2-terminal residues of the α subunits were omitted from the atomic models and as difference electron density maps calculated with deoxy HbA phases and $|F_{mutant} - F_{HbA}|$ magnitudes. The α+M and αV1M refinements were completed by an additional 15 cycles of restrained least-squares refinement. The final α+M structure has an *R* value of 0.170 and an R_{free} value of 0.223. The final αV1M structure has *R* and R_{free} values of 0.163 and 0.214, respectively. In both refined structures, good stereochemistry is indicated by root-mean-square (rms) deviations from bond lengths of less that 0.02 Å and by rms deviations from ideal bond angles of less than 1.8°.

RESULTS

Isolation and Functional Characterization of E. coli Expressed Hemoglobin. The hemoglobin expressed in *E. coli* was obtained from the cell lysate and isolated by chromatography on DEAE cellulose as described under Experimental Procedures. After removal of the CO by photolysis in the presence of O_2, this hemoglobin was examined kinetically. In Figure 2, the time course of the combination of CO with the deoxygenated *E. coli* expressed hemoglobin is compared to that for normal human hemoglobin A. Normal human deoxyhemoglobin reacts with CO with an autocatalytic reaction, the rate of which increases as the reaction proceeds (*28*). The expressed hemoglobin reacts with CO more rapidly than the normal human protein, and the reaction is heterogeneous, requiring a sum of more than one exponential function for a reasonable fit. This result is in agreement with the report of Hernan and Sligar (*7*).

Fractionation of E. coli Expressed Hemoglobin. The *E. coli* expressed hemoglobin, which had been isolated by DE52 chromatography, was subjected to HPLC fractionation using sulfopropyl (SP) and DEAE HPLC columns. HPLC fractionation on the SP resin resulted in only a modest improvement in the kinetic heterogeneity of the sample (data not shown). On the other hand, as shown in Figure 2, HPLC fractionation with the DEAE column resulted in a consider-

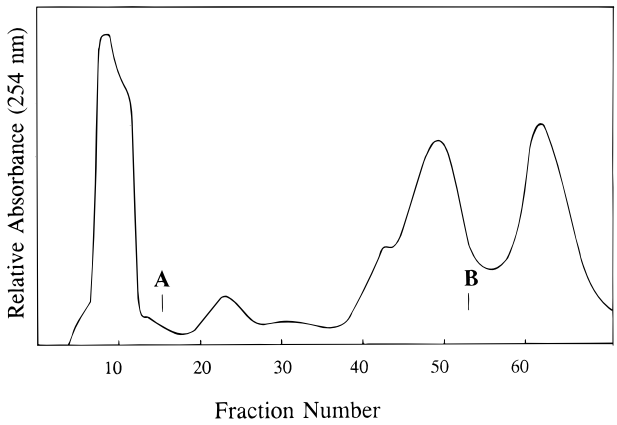

FIGURE 3: Fractionation of α and *β* globins of *E. coli* expressed hemoglobin on CM Sepharose using urea buffers (see Experimental Procedures). Elution was initially carried out with the pH 6.7 urea starting buffer. At position A on the Fraction Number axis gradient I, from pH 6.7 urea buffer to 0.1 M NaCl in the same buffer was initiated. At position B, gradient I was complete and gradient II, from 0.1 to 0.2 M NaCl, was started.

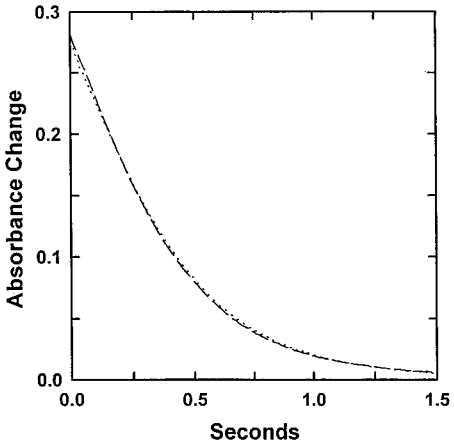

FIGURE 4: Kinetics of CO combination with HbA (− −) and reassembled *E. coli* expressed hemoglobin, αV1M−*β*V1M, (···). Reactions were carried out at 20 °C in 0.1 M HCl/bisTris buffer (pH 7).

able improvement in kinetic properties if only the central section of the single, major peak was taken, indicating that a partial separation of functionally dissimilar components was occurring. Multiple HPLC fractionations on the DEAE column often resulted in a kinetically homogeneous sample but never in a sample exhibiting autocatalytic CO combination kinetics. However, such fractionation reduced the final yield to an unusably low level, and the quality of the product obtained after a given number of purification cycles was not reproducible. This was unacceptable, because the correct functional behavior of a mutant protein is generally unknown and there is no standard by which to judge the adequacy of the fractionation of such a material when electrophoretic purity can accompany functional heterogeneity.

Shen et al. (*6*) reported that hemoglobin synthesized in a different *E. coli* coexpression system exhibited heterogeneity by NMR spectroscopy. They found that this spectroscopic heterogeneity could be eliminated by converting the hemoglobin to the ferric state and then to the ferrous deoxygenated state, followed by reaction with either CO or oxygen. We find that similar treatment of our *E. coli* produced hemoglobin has no significant effect on the kinetic heterogeneity we observe.

Complete Disassembly and Reassembly of E. coli Expressed Hemoglobin. Hernan and Sligar (*7*) had already demonstrated that separating the α and *β* subunits of an *E. coli* expressed hemoglobin and recombining them significantly reduced but failed to eliminate the functional heterogeneity of the sample. With the elimination of that procedure as a means of obtaining reproducibly homogeneous hemoglobin preparations, it was decided to explore the effects of completely disassembling the protein by removing the hemes, separating the α and *β* globins, and then reassembling the tetrameric protein from its individual components. The *E. coli* expressed hemoglobin was treated with acid acetone, and the resulting globin pellet was dissolved in urea buffer and fractionated in urea buffers on a CM Sepharose column (see Experimental Procedures). The profile of elution from the Sepharose column is shown in Figure 3. A significant impurity emerges with the starting buffer. There follow a few more small peaks of impurities and then the *β* globin peak, followed by the α globin peak. On SDS PAGE, both of the α and *β* globin fractions migrate as single bands of correct molecular weight (data not shown). SDS PAGE of the sample prior to fractionation on CM Sepharose revealed multiple nonglobin protein bands. Chromatography in urea buffers on CM Sepharose separates the α and *β* globins from one another, as well as from these impurities.

Functional Properties Resulting from Reassembly of E. coli Expressed Hemoglobin. The purified α and *β* globins were permitted to refold and react with one another in the presence of cyanohemin as described in Experimental Procedures. After completion of the assembly reaction, the reassembled hemoglobin was reduced anaerobically and purified by fractionation on a DE52 column (see Experimental Procedures). The resulting αV1M−*β*V1M hemoglobin migrated as a single component on PAGE, was spectrally indistinguishable from normal HbA, and exhibited normal stability with respect to auto-oxidation. The kinetics of CO combination with this hemoglobin were compared to those for HbA, and the result is shown in Figure 4. The time courses for the two reactions are almost superimposable. Oxygenation isotherms for the reassembled αV1M−*β*V1M hemoglobin were measured at four hemoglobin concentrations: 0.97, 10.6, 33.8, and 101 *μ*M in heme equivalents. These data along with the value of $^0\Delta G_2$ as determined by the haptoglobin technique (see Experimental Procedures) were used to calculate the complete linkage map by means of global regression analysis. In Figure 5, this map for αV1M−*β*V1M hemoglobin is compared to the same map for normal human HbA. The two linkage maps are not identical, but within error, they are remarkably similar. Only two independent parameters differ significantly. ΔG_{41}, the standard free energy for binding of the first oxygen onto the hemoglobin tetramer, is more negative for the reconstructed hemoglobin than for HbA. $^4\Delta G_2$, the standard free energy for association of the two fully oxygenated α*β* dimers to form the hemoglobin tetramer, was found to be less negative for the reconstructed hemoglobin. Although the differences between fitted values of ΔG_{43} were also large, their even larger statistical uncertainties render them indistinguishable.

Preparation and Functional Properties of αV1M and α+M Hemoglobins. The procedure developed for the reassembly of an *E. coli* expressed hemoglobin results in the

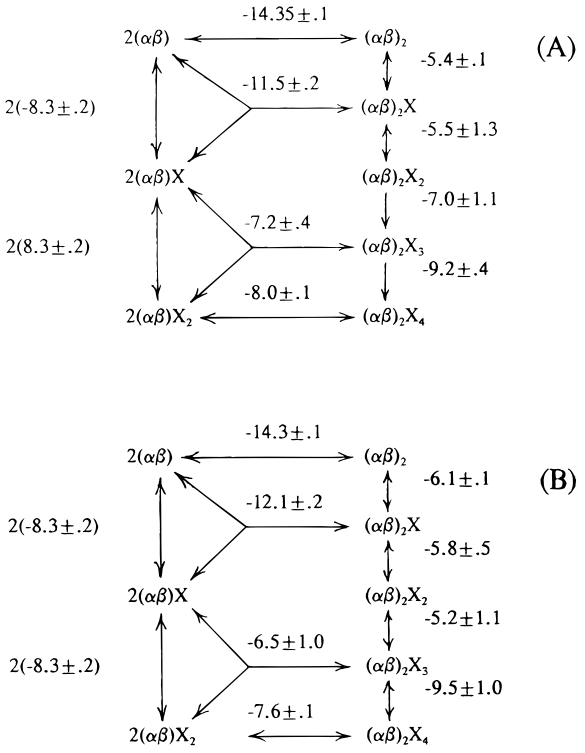

FIGURE 5: Linkage diagrams for cooperative O_2 binding and subunit assembly for (A) normal human hemoglobin (*17*) and (B) recombinant mutant αV1M. Concentrations examined for B are 0.97, 10.6, 33.8, and 101 μM heme.

Table 1: Subunit Assembly and Oxygenation Free Energies for Normal HbA and Recombinant Hemoglobins

sample	ΔG_4/kcal	$^4\Delta G_2$/kcal	P_{Med}/Torr	n_{Max}
HbA	−27.09 ± 0.05	−8.05 ± 0.1	5.1 ± 0.1	3.1
αV1M−βV1M	−26.69 ± 0.05	−7.59 ± 0.1	6.4 ± 0.1	2.7 ± 0.2
αV1M	−27.2 ± 0.3	−7.7 ± 0.7	5.1 ± 0.7	2.9 ± 0.2
α1+M	−28.4 ± 0.2	−8.6 ± 0.6	3.0 ± 0.1	2.9 ± 0.2

isolation of the separate α and β globin proteins. Because the majority of the hemoglobin variants to be prepared by this coexpression system will involve mutations only on the α chain, an alternative means of reassembly is to combine the mutant α globin with its normal, heme-containing partner β chain and cyanohemin. This alternative is attractive because it results in a 2−3-fold increase in the final yield of product. The product of this procedure is αV1M hemoglobin, which is assembled from the αV1M globin, normal β chains, and heme. To examine the effect of modifying the NH_2-terminal sequence of the α chain, the α+M globin, with an extra methionine before the normal N-terminal valine in the amino acid sequence, was also coexpressed with the βV1M globin and isolated and combined with normal β chains and heme to form α+M hemoglobin. The equilibrium and kinetic properties of these two reassembled hemoglobins were compared to those of HbA and αV1M−βV1M hemoglobin.

Oxygenation isotherms were measured for αV1M and α+M hemoglobins at three hemoglobin concentrations. These data were sufficient for the determination of both ΔG_4, the overall standard free energy associated with the binding of all four oxygen molecules to the hemoglobin tetramer, and $^4\Delta G_2$ for each hemoglobin. These values are presented in Table 1, along with the same parameters for HbA and αV1M−βV1M hemoglobin. The median oxygen partial pressure, P_{med}, and the maximum Hill coefficient, n_{max}, are also given for comparison. All three of the assembled mutant hemoglobins were found to exhibit highly cooperative oxygen binding. The reassembled αV1M−βV1M hemoglobin had a 20% lower oxygen affinity than HbA under these experimental conditions, and α+M hemoglobin had a higher than normal oxygen affinity. No significance difference was observed between αV1M hemoglobin and HbA. Two different kinetic measurements were carried out; the kinetics of CO combination with the deoxygenated hemoglobin molecule, as measured by rapid mixing, and the kinetics of CO recombination with hemoglobin following the complete photolysis of the CO-saturated hemoglobin molecule. The second-order rate constants for the combination of CO with the deoxygenated derivatives of αV1M−βV1M, αV1M, and α+M hemoglobins along with those for HbA in the absence and presence of 0.1 mM IHP are presented in Table 2. For αV1M and HbA, the dependencies of these rate constants on pH are reported. Given an average standard error of these rate constants of ±10%, none of the kinetic differences among these hemoglobins appear to be significant. The second-order rate constants for the recombination of CO following flash photolysis in the absence and presence of 0.1 mM IHP at pH 7 are listed in Table 3. Two rate constants are reported for each experimental condition. At the hemoglobin concentration used in these measurements, CO-saturated hemoglobin is partially dissociated into αβ dimers. The faster of the two recombination reactions is the reaction of αβ dimers with CO. The slower kinetic process represents the tetrameric hemoglobin molecules. The percentages quoted refer to the fractional contribution of the two kinetic components to the overall reaction and give a measure of the degree of dissociation of the CO-saturated hemoglobin molecule into αβ dimers. From these data, the reassembled αV1M−βV1M hemoglobin is somewhat more dissociated than HbA when saturated with CO, αV1M hemoglobin is similar to HbA, and α+M hemoglobin is somewhat less dissociated.

Table 2: Second-Order Rate Constants for the Combination of CO with HbA and Reconstituted Hemoglobins (μM^{-1} s^{-1})

pH	IHP	HbA	αV1M−βV1M	αV1M	α+M
6	−	0.16 A		0.16 A	
	+	0.09 A		0.1 A	
6.5	−	0.16 A		0.17 A	
	+	0.1 A		0.1 A	
7	−	0.16 A	0.16 A	0.17 A	0.14 A
	+	0.08 A	0.09 A	0.09 A	0.08 A
7.5	−	0.21 A		0.24 A	
	+	0.12 A		0.11 A	
8	−	0.26 A		0.22 A	
	+	0.13 A		0.11 A	

Table 3: Second-Order Rate Constants for the Recombination of CO following Complete Photodissociation (μM^{-1} s^{-1})

pH	IHP	HbA	αV1M−βV1M	αV1M	α+M
7	−	5.2 (68%)	3,8 (76%)	4.6 (66%)	5.2 (49%)
		0.13 (32%)	0.12 (24%)	0.15 (34%)	0.14 (51%)
7	+	4.8 (32%)	3.4 (45%)	3.8 (36%)	4.3 (25%)
		0.08 (68%)	0.08 (55%)	0.07 (64%)	0.07 (75%)

X-ray Crystal Structure of Deoxy αV1M. Analysis of the refined αV1M crystal structure indicates that the valine−methionine substitution has little effect on the structure of

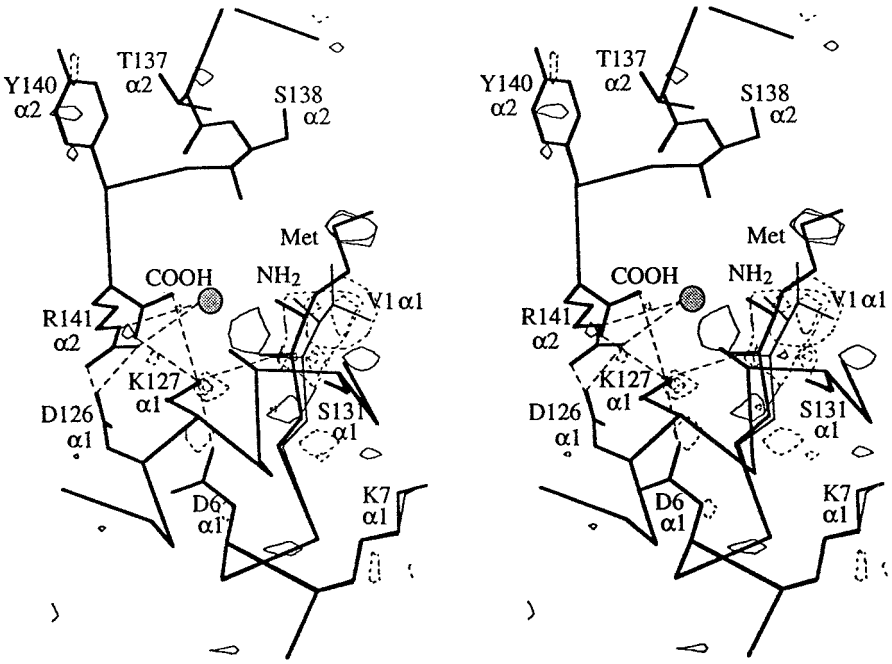

FIGURE 6: Stereoplot showing the α1−α2 interfaces of deoxy αV1M Hb (thick bonds) and deoxy HbA (thin bonds). Overlaid in the atomic models is the deoxy αV1M minus deoxy HbA difference map that was calculated with deoxy HbA phases and $|F_{\alpha V1M} - F_{HbA}|$ magnitudes. Positive difference contours (solid lines) and negative contours (dashed lines) are drawn at increments of 2 times the rms density of the map. The network of polar interactions between Val1α, Asp6α, Asp126α, and Lys127α on the α1 subunit and the COOH-terminal Arg141α of the α2 subunit is shown as dashed lines. The filled circle located next to the side chain of Arg141α indicates the position of a bound solvent molecule that is present in both deoxy HbA and deoxy αV1M. Although the solvent molecule was refined as a water molecule, the area where this solvent molecule is bound is thought to be a chloride binding site (*30*, *31*).

the $\alpha_2\beta_2$ hemoglobin tetramer. Superposition analysis (see ref *29*) reveals no change in quaternary structure or tertiary structure. Specifically, relative to deoxy HbA, ∼0.2 Å movements are observed for the main chain atoms of the α subunit and β subunit NH_2-terminal residues of αV1M and the movement of main chain atoms for the remainder of the structure averages 0.03 Å and never exceeds 0.1 Å (data not shown). Small increases in temperature factors (from ∼35 to 45 Å^2) relative to deoxy HbA are also observed at the α NH_2-terminal of αV1M. Some of these very small changes in position and mobility probably represent random changes due to data "overfitting", and this is consistent with the failure of these changes to cross validate with a decrease in the R_{free}^{local} value (*29*).

In Figure 6, the deoxy αV1M atomic model (thick bonds) is superimposed on the deoxy HbA atomic model (thin bonds). Except for the small difference in the position of NH_2-terminal residues noted above, the two models are indistinguishable. Also shown in Figure 6 is the αV1M minus deoxy HbA difference electron density map, which is free of the refinement bias mentioned above. The negative difference density situated over the main chain atoms of Val1α is consistent with a small increase in mobility, suggesting that at least some of the small increase in the refined temperature factors of the α subunit NH_2-termini are mutation-related. In addition, the negative difference density located over one of the Val1α methyl groups is consistent with the loss of the β-branched valine side chain. The absence of significant positive difference electron density at the 2σ level indicates that the methionine side chain is highly disordered. However, by contouring the difference map at lower levels, the methionine side chain could be positioned so that the electron-dense S_δ atom was located in the region of highest positive difference density. When refined in this position, the side chain of Met1α makes no significant contact with neighboring residues, and its average atomic temperature factor is 106 Å^2 for the α1 subunit and 117 Å^2 for the α2 subunit.

X-ray Crystal Structure of Deoxy α+*M.* As was the case for the αV1M mutation, superposition analysis of the refined deoxy α+M atomic model revealed no significant mutation-induced changes in quaternary or tertiary structure relative to deoxy HbA. The largest main chain atomic movements occur at the α subunit and β subunit NH_2-termini and range from ∼0.2 to ∼0.3 Å, and there are very small increases in the temperature factors of the α subunit NH_2-termini. However, as in the case of the deoxy αV1M structure, these small structural changes fail to produce a significant cross-validation signal by decreasing the R_{free}^{local} value.

Whereas no significant positive difference density is observed for the methionine side chain in the deoxy αV1M minus deoxy HbA difference electron density map, the deoxy α+M minus deoxy HbA difference map (Figure 7) clearly shows positive difference density for the NH_2-terminal methionine residue. This positive difference density for the NH_2-terminal methionine of the α1 subunit is oriented away from residues Thr137, Ser138, and Tyr140 of the α2 subunit and toward the side chain Arg141 of the α2 subunit and residue Ser131 of the α1 subunit (Figure 7). When fit into this difference electron density, none of the methionine side chain atoms were within 4.0 Å of any of these residues. Because the methionine side chain cannot make significant van der Waals contacts, it is highly mobile, and this is reflected in high atomic-temperature factors that average ∼110 Å^2 in the α1 subunit and ∼90 Å^2 in the α2 subunit. However, a negative difference density peak clearly indicates

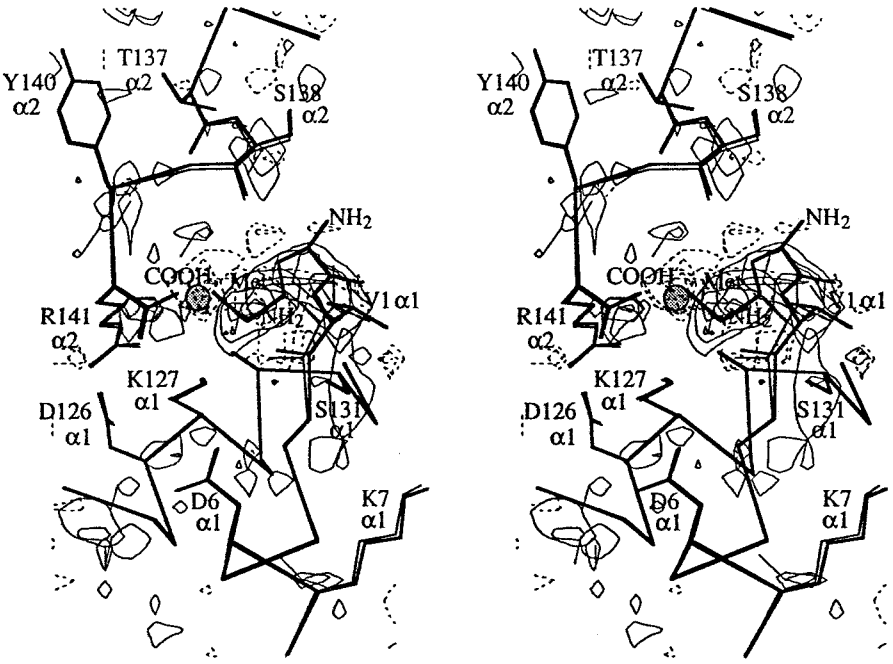

FIGURE 7: Stereoplot showing the α1−α2 interfaces of deoxy α+M (thick bonds), deoxy HbA (thin bonds), and the deoxy α+M minus deoxy HbA difference map that was calculated with deoxy HbA phases and $|F_{\alpha+M} - F_{HbA}|$ magnitudes. Positive difference contours (solid lines) and negative contours (dashed lines) are drawn at increments of 2 times the rms density of the map. Negative difference contours indicate that the methionine side chain displaces the water (or chloride ion) that normally interacts with the side chain of Arg141α.

that the methionine side chain displaces a solvent molecule (water or disordered chloride ion) that is normally bound to Arg141α2 in deoxy HbA (Figure 7).

Similarities between the deoxy structures of α+M and the naturally occurring mutant, Hb Thionville, suggest that the slightly increased oxygen affinity of the α+M mutant may be due to a decrease in the allosteric effect of the chloride ion. Vassuer et al. (*30*) have demonstrated that in Hb Thionville the NH_2-terminal valine of the α subunit is mutated to glutamic acid and the initiator methionine is retained and acetylated. Two conformations for the additional NH_2-terminal-acetylated methionine are observed in deoxy Hb Thionville (see Figures 4 and 5 of ref *30*), and one of these corresponds to the conformation of the NH_2-terminal methionine in deoxy α+M. Significantly, the solvent molecule that is displaced in deoxy α+M is also displaced in deoxy Hb Thionville (see Figure 4D of ref *30*). On the basis of a number of X-ray crystallographic studies as well as solution studies (*30* and *31* and references therein), the area where this solvent molecule is bound is thought to be a chloride binding site. Vassuer et al. (*30*) attributed the reduced chloride effect in Hb Thionville to the elimination of this anion binding site. Similarly, the loss of this solvent binding site in deoxy α+M may be the basis for the slight increase in the oxygen affinity of α+M.

DISCUSSION

The goal of expressing human hemoglobin genes in microorganisms and subjecting them to site-directed mutagenesis is the production of mutants having properties that duplicate those which would be found if these mutant molecules were synthesized in a human red cell. The coexpressed hemoglobin examined in this study is a double mutant, αV1M−βV1M. When this coexpressed hemoglobin was found to be functionally heterogeneous, it appeared likely that the assembly of hemoglobin within the *E. coli* organism was associated with the formation of an abnormal product. It had previously been shown that when hemoglobin is assembled from normal human α chains, heme, and βV1M globin produced by the β globin expression system of Hernan et al. (*4*), the resulting tetrameric hemoglobin is functionally homogeneous with properties that closely approximate those of normal human HbA. At the same time, it was not expected that replacement of the NH_2-terminal valine of the α chain with methionine would result in a significant perturbation in the properties of the protein. The suspicion that the observed kinetic heterogeneity resulted from assembly within *E. coli* has been confirmed by the identification of a procedure for the elimination of the functional abnormalities.

When the *E. coli* expressed αV1M−βV1M hemoglobin is reassembled from its constituent α and β globins and hemin, the tetrameric hemoglobin which results is functionally homogeneous and the equilibria and kinetics of its reactions with ligands are similar to those of HbA. Although the overall oxygen affinity is somewhat lower than that of HbA and as a result of thermodynamic linkage the ligand-saturated molecule is slightly more dissociated into αβ dimers, the pattern of affinity changes which accompanies the stepwise binding of oxygen to the hemoglobin tetramer is found to mimic that of HbA. Only the binding of the first molecule of oxygen has a free energy significantly different from that of the normal human protein. The oxygen affinity at this step is found to be greater than normal. The latter observation is surprising given the similarity of the kinetics of CO binding to deoxygenated HbA and to the reassembled αV1M−βV1M hemoglobin. However, close examination of the time courses of the CO combination reactions in Figure 2 reveals that the initial rate of the reaction of CO with the reassembled hemoglobin is somewhat greater than that for HbA. This may indicate that the reassembled hemoglobin

still contains a small amount of relatively high-affinity contaminant. Such a contaminant would have its greatest effect on ΔG_{41} and result in an overestimate of the affinity of this step in the saturation process. However, the data are insufficient to establish the presence of such an impurity. The greater than normal dissociation of αV1M−βV1M into αβ dimers at pH 7.4 as estimated from equilibrium thermodynamic analysis is consistent with the kinetics of CO recombination following flash photolysis at pH 7. The contribution of the rapid kinetic phase to the overall reaction is greater for reassembled αV1M−βV1M than for HbA.

In a previous study, Doyle et al. (*10*) examined three hemoglobin variants with different NH_2-terminal constructs on their β chains: βV1M hemoglobin with the NH_2-terminal valine replaced by methionine, βV1A hemoglobin with an NH_2-terminal alanine, and β+M hemoglobin with an extra methionine at the N terminus followed by the normal valine residue. Of these three constructs, the βV1M most closely approximated the functional properties of HbA and exhibited the least structural perturbation as observed by X-ray crystallography. We have examined hemoglobins assembled from the αV1M and α+M constructs of α globin and normal β chains. The functional properties of these two mutants have been characterized less completely than those of reassembled αV1M−βV1M hemoglobin but sufficiently to demonstrate, as expected, that both mutants are relatively normal, cooperative hemoglobin molecules. Within error, the equilibrium and kinetic properties of αV1M hemoglobin are indistinguishable from those of HbA, but α+M exhibits an increased oxygen affinity and, when saturated with ligand, has a reduced tendency to dissociate into αβ dimers. Once again, the kinetics of CO recombination following flash photolysis reinforce the thermodynamic measurements. The percentage of the overall recombination reaction associated with the high rate constant, i.e., the percentage of the hemes in αβ dimers, is least for the α+M hemoglobin whereas the αV1M hemoglobin differs little from HbA. The X-ray crystal structures of deoxy αV1M and deoxy α+M are consistent with the kinetic and thermodynamic measurements in that both mutant structures are highly isomorphous with the structure of deoxy HbA. The structure of the deoxy αV1M variant is essentially identical to the structure of deoxy HbA, and only a subtle change in the solvation of the α1−α2 interface is detected in the deoxy α+M mutant.

To be useful, a procedure for production of hemoglobin variants must result in sufficient material in a single preparation to carry out a wide array of studies. In this way, results from different studies can be conducted on the identical material and direct comparison is possible. In general, at least 100 mg is required for a relatively complete functional and structural characterization of a mutant (*8*, *18*, *29*, *32*). It was this need that made purification by multiple chromatographic steps with their concomitant losses unsatisfactory, and which also prompted the examination of the reassembly of hemoglobin from *E. coli* expressed α globin, heme, and β chains. The yield in the latter procedure is far better than when hemoglobin is assembled entirely from subunit globins and heme. Because this coexpression system will be used primarily for the production of α chain variants of hemoglobin, the alternative assembly procedure with its higher yield is the most attractive. Although the differences between α+M and αV1M hemoglobins are not great, the near identity, both functionally and structurally, of the latter mutant and HbA makes αV1M the obvious choice for the hemoglobin construct with which to examine the effects of other α chain mutations on the properties of the hemoglobin molecule.

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