Tetrameric Hemoglobin Expressed in Escherichia coli

EVIDENCE OF HETEROGENEOUS SUBUNIT ASSEMBLY*

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Recombinant \( \alpha_2\beta_2 \) tetrameric Hb expressed and assembled in Escherichia coli has been characterized extensively. Electrospray mass spectrometry and optical and electron paramagnetic resonance spectroscopy suggest that the overexpressed protein is identical to native human Hb. Although the functional properties of this recombinant Hb are nearly identical to native Hb, crucial differences exist between the two molecules. The recombinant Hb expressed in E. coli has a lower Hill coefficient even though oxygen equilibrium binding studies indicate cooperative binding. The most significant difference observed between the recombinant and native Hb is the loss of oxygen affinity regulation by 2,3-diphosphoglycerate and protons. CO binding to the native Hb is the loss of oxygen affinity regulation by 2,3-diphosphoglycerate and protons. CO binding to the recombinant Hb produces a protein that is subject to regulation by allosteric effectors. Furthermore, CO binding to the deoxy tetramer was found to be biphasic with both phases sensitive to the presence of allosteric effectors. The recombinant chains were isolated, and the ligand binding properties demonstrated that the recombinant chains behave in a similar fashion to native \( \alpha \) and \( \beta \) chain. To investigate whether the chains were capable of forming a well behaved tetramer, the isolated chains were reassembled into a tetramer and purified to homogeneity. Oxygen binding properties of the reassembled recombinant Hb now show an increased Hill coefficient of 2.5, close to, but still slightly lower than, that observed for native Hb. Additionally, reassembly of recombinant Hb produces a protein that is subject to regulation by allosteric effectors. Furthermore, CO binding to the reassembled recombinant deoxy tetramer was found to be monophasic under all conditions.

Hb has long been studied as a model compound for many biochemical phenomena and continues to be the object of intense work to elucidate the molecular details of protein-protein recognition, allosteric regulation, ligand binding and dynamics, spectroscopy, energetics of cooperativity, and structure-function relationships (1–3). X-ray structures of liganded (R), unliganded (T) states, and with several intermediate species have been solved to high resolution and have provided much information on the detailed characterization of the mechanism of action by Hb (4–7). In addition to mechanistic and structure-function studies, Hb has grown in popularity for use in clinical applications as a potential blood substitute (8). These efforts, along with the possibility of engineering specific and novel properties into the molecule, have prompted the development of many recombinant Hb expression systems. We have investigated a purified recombinant human Hb using a coexpressing system in Escherichia coli (9) in order to determine whether a completely assembled recombinant tetrameric Hb constitutively expressed in E. coli is fully functional.

The first expression of individual human Hb chains in E. coli was reported by Nagai and Thøgerson (10). Their expression system involved producing \( \beta \)-globin as an insoluble fusion protein, which was solubilized, purified, and cleaved with factor Xa to produce the correct N terminus. The \( \beta \)-globin was then reconstituted and refolded in the presence of native \( \alpha \) chain to produce a well behaved cooperative semi-recombinant tetramer (11). Further characterization of this protein including X-ray crystallography (12), Raman spectroscopy, and CO combination kinetics showed that the behavior of this semirecombinant Hb was identical to that of native Hb (13). Individual recombinant \( \alpha \)-globin has also been expressed in a similar fashion and refolded with native \( \beta \)-globin to produce a well behaved semi-recombinant tetrameric Hb (14). Recently, a modified fusion protein system under chemical induction was used to produce a recombinant \( \beta \)-globin which was then reconstituted into a tetramer using a simplified method to produce a functional, semi-recombinant tetrameric Hb (15). Our previous report has shown that the \( \beta \) chain can also be expressed from cDNA clones as insoluble aggregates using a T7 promoter without the use of a fusion protein, thus circumventing some of the laborious problems associated with a fusion protein expression system (9). Unlike yeast (16, 17) and the fusion protein expression systems, this construction leaves the initiator methionine on the N terminus. Characterization of three N-terminal \( \beta \) chain mutants produced in E. coli using the T7 expression system (\( \beta + \text{Met} \rightarrow \text{Ala} \), and \( \beta \text{Val} \rightarrow \text{Met} \)) has been reported (18). The addition of the initiator methionine at the N-terminal of \( \beta \) chain produced a good semirecombinant tetramer with only minor perturbations, but the removal of the valine at the second position (\( \beta \text{Val} \rightarrow \text{Ala} \)) produced a good semirecombinant tetramer with only minor perturbations, but the removal of the valine at the second position (\( \beta \text{Val} \rightarrow \text{Met} \)) produced a semi-recombinant tetramer that was almost identical in allosteric behavior, thermodynamic linkage, dimer-tetramer equilibrium, and CO combination kinetics. In addition, X-ray structures of these mutants indicated that the \( \beta \text{Val} \) mutation had the least amount of structural perturbation (19) and would serve as a good starting point for further mutagenic studies.

Expression of a complete recombinant soluble heme containing tetrameric Hb has recently been achieved by coexpressing both \( \alpha \) and \( \beta \) chains in E. coli (9, 20). Initial characterization of a soluble cytoplasmic heme-containing tetramer by coexpression of \( \alpha \) and \( \beta \) chains from one plasmid in E. coli has been performed (20). The protein produced in these studies co-migrated on an SDS-polyacrylamide gel electrophoresis with native Hb (HBA) and had similar absorption spectra and chromatographic behavior as HBA. However, oxygen equilibrium binding studies on this recombinant Hb showed a reduced Hill coefficient.

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coefficient, Bohr effect, and IHP1 effect compared to native Hb, presumably due to the presence of the initiator methionine. To circumvent the inability of E. coli to process the initiator methionine of both recombinant Hb chains, we have constructed N-terminal mutations for α and β chains. The mutations remove the second amino acid, valine, by removing the second codon triplet in the synthetic genes to produce a recombinant Hb with ^3^Val → Met and ^3^Val → Met (Hb des Val). These modifications to the N terminus should have minimal effect on a completely recombinant Hb since the allosteric role of these residues is primarily associated with the amino group rather than the side chain (25, 26). We have purified recombinant human Hb coexpressed in E. coli to homogeneity and examined the physical properties, using optical spectroscopy and EPR, and compared them to native Hb. Functional properties investigated include oxygen equilibrium binding to examine the oxygen affinity, cooperativity, pH and allosteric effects of organic phosphates on oxygen binding. Carbon monoxide binding to deoxygenated recombinant Hb was performed to examine the T-state properties and allosteric effects of organic phosphate and pH on CO binding kinetics. The integrity of the isolated chains were examined using flash photolysis and displacement reactions to determine their ligand binding properties. A completely recombinant tetrameric Hb was reassembled from these isolated chains, purified and its functional properties examined by measuring the kinetics of CO binding to deoxygenated Hb, allosteric effects of organic phosphate on the CO binding kinetics, and oxygen equilibrium binding in the presence and absence of allosteric effectors IHP, DPG, and protons. The CO combination kinetics provide an excellent means to probe the properties of the deoxy or T-state Hb and its response to allosteric regulation while the oxygen binding data provide a measure of cooperativity and response of the reassembled Hb to allosteric regulation by organic phosphate and pH.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions—** E. coli strain TB-1lara, Jlac-pro, Stlha, ϕ80lacZ15R-M1 was used for growth and expression of recombinant human Hb. Plasmids pHS471 and pWHS 486 (9) were used for construction of mutants. Small cultures were grown in LB media containing 200 µg/ml ampicillin in an incubator at 37 °C with 200 rpm agitation for approximately 16 h.

**Cassette Mutagenesis—** N-terminal α and β chain des Val mutants were constructed using cassette mutagenesis techniques (9). Two synthetic oligonucleotides which contained the deletion of the valine were inserted between the NcoI and SscI sites of the ϕ HindIII chain and the NcoI and HpaI sites of the β chain. Removal of the GTT codon introduced a GTT codon. The samples were concentrated under N2, then stored frozen at −20 °C until use.

**Hb Purification—** E. coli TB-1 strains containing Hb des Val were grown in 2 × YT medium with 0.2 g l-mannitol in 30-liter batches at the University of Illinois fermentation facility. The cells were harvested by centrifugation and stored at −70 °C. Cells were thawed over a stream of CO, then resuspended in 3 times (w/v) of CO saturated 25 mM Tris-HCl, pH 8.0, 2 mM EDTA. Lysozyme, RNase, and DNase were added to a final concentration of 4 mg/ml, 8 units/ml, and 80 units/ml, respectively, and the mixture was stirred at 4 °C for 1 h. The mixture was centrifuged at 10,000 × g for 15 min at 4 °C, the pellet was discarded, and the red supernatant was adjusted to pH 6.5 with 20 mM NaH2PO4, then diluted 1:1 with CO-saturated H2O2. The mixture was loaded onto a CM-cellulose column equilibrated with CO-saturated 10 mM sodium phosphate, pH 6.5, 1 mM EDTA. The column was washed with starting buffer, and the Hb was eluted with CO-saturated 20 mM sodium phosphate, pH 8.0, 1 mM EDTA. The major peak was collected, concentrated under N2 using an Amicon P-30 membrane. The material was further purified by ion exchange HPLC using a fast protein liquid chromatography system (Pharmacia Biotech Inc.) equipped with an Altex SP-5PW high performance cation exchange column (2.15 × 15 cm). Hb was loaded onto the column equilibrated with 25 mM sodium phosphate, pH 6.0, and eluted with a linear gradient from 25 mM sodium phosphate, pH 6.0, to 25 mM Tris-HCl, pH 8.6, at a flow rate of 5 ml/min. Elution was monitored at 405 nm, and the major peak was collected and concentrated as before. The CO was removed by illuminating the protein sample on ice under 1 atm of oxygen. The material was exchanged into 50 mM Tris-HCl, pH 7.4, using a G-25 column, and stored at −70 °C until use. Native Hb was purified from fresh red blood cells. Both recombinant and native α and β chains were isolated using the method of Bucci (22).

**Reconstitution of Tetrameric Hb—** Isolated recombinant α^-3^ in CO-saturated 50 mM Tris-HCl, pH 7.4, was mixed with 1.2 molar excess recombinant β^-3^ CO bound and then incubated overnight at 4 °C. The tetrameric Hb was purified from excess β chain by high performance ion exchange chromatography as described above. The samples were collected and exchanged into 50 mM Tris-HCl using a G-25 column. The CO was removed by illuminating the stirring sample under 1 atm of O2 at 4 °C. The samples were concentrated under N2, then stored frozen until use.

**Electrophoresis—** UV-visible spectra of purified recombinant human Hb in 50 mM Tris-HCl, pH 7.4, was recorded at ambient temperature using a Hewlett Packard 8450A U/VIS spectrophotometer. Electron Paramagnetic Resonance—Electron paramagnetic resonance spectroscopy of oxidized native and recombinant Hb was performed at a concentration of 100 µM in 50 mM Tris-HCl, pH 8.9, on a Bruker ER 200 DESR spectrometer (modulation frequency, 100 kHz; modulation amplitude, 5 G; power 16 dB (5 mW); microwave frequency, 9.2982 GHz at 7 K).

**Mass Spectrometry—** Electrospray mass spectrometry of purified recombinant and native Hb were obtained at the University of Illinois Mass Spectrometry Facility. The proteins were dissolved in a 50:50 v/v acetonitrile/H2O solution containing 0.1% formic acid at a final concentration of 10 pmol/µl.

**Oxygen Binding Properties—** Oxygen binding properties of recombinant and native human Hb were recorded using a continuing recording instrument similar to that described by Imai et al. (23) and Imai and Yonetani (24). The sample cell was constructed from plexiglass, fitted with two quartz windows, and contained a ventilation system for the exchange of humidified gases. A stirring motor was mounted underneath the cell. The cell was fitted with a Lauda K-2/2R circulating water bath at 25 ± 0.1 °C and monitored with a thermostat. A YSI 5331 Clark type oxygen probe was used to monitor the oxygen tension of the Hb solutions. The polarization voltage of 0.8 V, the current output of the electrode, current to voltage conversion, and voltage amplification were supplied by a Bioanalytical systems CV-27 voltamograph. The cell and stirring motor were mounted on a Varian DMS-102 spectrophotometer, and the change in absorbance at 560 nm was measured. Deoxy- oxygenization was carried out by flushing the stirred sample with humidified nitrogen which had been passed through an in-line oxygen scrubber. Data for deoxy- gination of Hb samples was collected at intervals of 10 s per point with a typical deoxygenation taking 40–45 min. The oxygen binding curve and absorbance recorded simultaneously as voltampere using a Hewlett Packard 3840 multimeters interfaced with a personal computer. The raw data were converted to oxygen pressure and absorbance using software written in-house. Samples were prepared from frozen stocks and diluted to 60 µM heme in 50 mM Tris-HCl (pH = 7.4) 100 mM Chloride concentration was adjusted by the addition of NaCl. DPG and IHP were added from buffered solutions. An enzymatic reduction system described by Hayashi et al. (25) was employed to reduce the amount of metHb formation.

**CO Recombination Kinetics—** CO recombination kinetics of isolated
recombinant and native chains were used to measure the association rates of CO to isolated α and β chains. Samples were prepared from stock solutions by dilution into 0.1 M NaH₂PO₄, pH 7.0. Dithionite was added anaerobically to a final concentration of 100 μM to maintain anaerobic conditions. CO was added in 10-fold excess to ensure pseudo first order conditions. The CO was photolyzed using two Sunpak 344 photographic strobes equipped with a thyristor quenching device. The discharge was a square wave with a 0.1-ms rise and fall time and a total flash duration of 0.5 ms. To reduce interference from the flash lamps during monitoring of the observation beam, the flash radiation was filtered using a 450-nm cut-off filter. Absorbance at 420 nm was recorded starting 1 ms after initiation of the flash. The raw data were collected and then stored using a Nicolet 3091 storage oscilloscope. The raw data were converted to absorbance using software developed in-house. The data were fit to a single exponential decay to obtain the observed rates.

**CO Displacement Reactions**—The off rate of carbon monoxide from isolated recombinant and native α and β chains was determined by a displacement reaction with NO. A saturated solution of NO was mixed anaerobically with an equal volume of a 20 μM protein solution. The final concentration of NO was 1 mM, and the final concentration of protein was 10 μM. The absorbance at 420 nm was recorded using a Hitachi 3300 spectrophotometer and the observed rates were determined by fitting the traces to a single exponential decay.

**CO Combination Kinetics**—Stock solutions of native and recombinant Hb were diluted in 50 mM Tris-HCl at the appropriate pH to a final concentration between 4–5 μM heme before mixing. The concentration of chloride ion was adjusted with NaCl to give a final concentration of 0.1 M. While there were differences in the amount of sodium in solution the amount of chloride, the stronger effector, remained constant under all conditions (26, 27). Hb was deoxygenated and dithionite was added anaerobically to a final concentration of 2 mM to ensure complete anaerobicity.

A Kinetic Instruments (Bethesda, MD) stopped flow equipped with a 2-cm path cell and a 2-ms dead time was used. The temperature was controlled at 20 ± 0.1 °C with a Haake F3 circulating water bath and monitored with a thermistor. The absorbance changes at 420 and 435 nm were measured and stored as raw voltages on a Nicolet 3901 storage oscilloscope. The conversion of voltage to absorbance was performed using software written in our laboratory. The kinetic traces for native Hb were fitted with a single exponential to determine the observed rate. Recombinant Hb was fitted with either a single exponential or the sum of two exponentials.

**RESULTS**

Recombinant Hb—The Hb des Val construction was made as described under "Experimental Procedures." The mutation to β des Val was used based upon the results reported by Doyle et al. (18) who showed that this mutation at the N-terminal of β-globin assembled into αβββ Hb with functional properties identical to that of human Hb. The same valine to methionine substitution was constructed for the α chain with the idea that, here too, only minor perturbations in structure and function would occur. E. coli TB-1 cell harboring the plasmid Hb des Val was expressed as described by Hernan et al. (9) and was grown in a 30-liter fermentor. Holo-tetrameric human Hb coexpressed in E. coli on a pUC plasmid under control of the lac promoter yields 5-10% of the total soluble protein expressed. This expression system produces Hb constitutively and incorporates endogenous heme to form an assembled tetrameric recombinant Hb. Purification of Hb was facilitated by both the overexpression and the fact that the tetrameric Hb is less acidic than a majority of the E. coli proteins. CM-cellulose chromatography produced a product that was approximately 90% pure by SDS-polyacrylamide gel electrophoresis. High performance ion exchange chromatography removed minor contaminants that are unable to be resolved using conventional chromatography. This protocol produced a Hb that migrated as a single band on an SDS-polyacrylamide gel electrophoresis with an apparent molecular weight identical to that of native Hb. Additional characterization of recombinant Hb was necessary to determine if proteolytic degradation or N-terminal processing had occurred. Confirmation of the correct composition and sequence was performed using phenylthiohydantoin-derivative analysis (data not shown), and 10 cycles of Edman degradation N-terminal protein sequencing produced the sequence of Met, LeuHis, Ser/Leu, Pro/Thr, Ala/Pro, Asp/Glu, Lys/Glu, Thr/Lys, Asn/Ser, Val/Ala. In addition, heme stoichiometry was determined using a pyridine hemochromagen assay (28), and the ratio was found to be 4.1 heme to protein. Both native and recombinant Hb were further analyzed by electrospray mass spectrometry to determine atomic mass. The mass spectra shown in Fig. 1 shows two mass peaks of molecular weight 15,126 and 15,868 for native Hb which correspond to α and β chains, respectively. Recombinant Hb also produced two mass peaks with a molecular weight of 15,158 and 15,886, 32 atomic mass units greater than the native chains which is the expected difference in mass due to the substitutions of methionine for valine.

Optical spectroscopy of recombinant human Hb was used to probe the electronic environment of the porphyrin under different ligation conditions. The deoxy spectra has a Soret maximum at 432 nm and a broad visible band at 556 nm. The oxy Hb has a Soret maximum at 415 and 426 and a band at 540 nm. Carbon monoxide bound recombinant Hb has a Soret at 419 nm and α and β bands at 540 and 576 nm. Carbon monoxide bound recombinant Hb has a Soret at 419 nm and α and β bands at 540 and 570 nm, respectively. The optical spectra of purified recombinant Hb is identical to native Hb and corresponds well with reported values (21).

EPR spectra of both native and recombinant Hb were recorded at 7 K in 50 mM Tris-HCl, pH 8.9. The g values of 5.8 and 2.0 correspond to the high spin aqua bound complex. The g values at 2.5, 2.17, and 1.8 are for the low spin hydroxy complex. These data suggest that the recombinant Hb has ligand binding states identical to that of native Hb.

Oxygen equilibrium binding curves of recombinant and native Hb were measured by the continuous method described under "Experimental Procedures." Oxygen isotherms were measured in 50 mM Tris-HCl [Cl⁻] = 0.1 M at 25 ± 0.1 °C. Fig.
Oxygen equilibrium isotherm of native and recombinant Hb. Conditions: 50 mM Tris-HCl, pH 7.4, \([\text{Cl}^-]\) = 0.1 M at 25 °C. Protein concentration was 60 μM heme.

Table I

| Parameter          | Native HbA | Recombinant HbA |
|--------------------|------------|-----------------|
| \(P_50\) (mm Hg)   | 5.3 ± 1    | 5.4 ± 1         |
| \(n_H\)            | 2.8        | 2.0             |
| DPG effect \(P_50\) (mm Hg) | 12.9 ± 1 | 4.6 ± 1        |
| \(n_H\)            | 2.8        | 1.6             |
| \(\Delta \log P_50\) ± 2 mM DPG | 0.4       | 0.0             |
| IHP effect \(P_50\) (mm Hg) | 43.9 ± 1 | 44.4 ± 1        |
| \(n_H\)            | 2.4        | 1.8             |
| \(\Delta \log P_50\) ± 1 mM IHP | 0.9       | 0.9             |
| Bohr effect \(\Delta \log P_50/\text{pH}\) | -0.5      | -0.1            |

Fig. 2. Oxygen binding parameters of native and recombinant hemoglobins

**Conditions:** 50 mM Tris or Bis-Tris [Cl\(^-\)] 0.1 M at 25°C.

**Fig. 3. Normalized time course of CO combination to deoxy recombinant Hb (○) and deoxy native Hb (▼) in the absence (A) and presence (B) of 2 mM IHP.** Observations were made using a 2-cm path length and monitoring at 420 nm. Solution conditions were 50 mM Bis-Tris, pH 7.0, [Cl\(^-\)] = 0.1 M at 20 °C. Hb concentration was 2.5 μM heme after mixing.

Table II

| Conditions: 50 mM Tris or Bis-Tris [Cl\(^-\)] = 0.1 M at 20 °C. |
|----------------------------------------------------------------|
| Rate constants for CO combination to recombinant and native hemoglobin |
| Recombinant Hb | Native Hb |
| \(k_{\text{on}}\) | \(k_{\text{off}}\) | \(k_{\text{on}}\) |
| \(\times 10^9 \text{ M}^{-1} \text{s}^{-1}\) | \(\times 10^5 \text{ M}^{-1} \text{s}^{-1}\) | \(\times 10^5 \text{ M}^{-1} \text{s}^{-1}\) |
| pH 6 | 2.60 ± 0.2 | 2.20 ± 0.16 | 1.34 ± 0.03 |
| pH 6 + IHP | 0.94 ± 0.18 | 0.43 ± 0.16 | 0.49 ± 0.05 |
| pH 7 | 3.15 ± 0.28 | 2.67 ± 0.25 | 1.45 ± 0.09 |
| pH 7 + IHP | 1.66 ± 0.12 | 1.91 ± 0.3 | 0.66 ± 0.08 |
| pH 8 | 3.29 ± 0.48 | 3.37 ± 0.57 | 3.06 ± 0.6 |
| pH 8 + IHP | 1.90 ± 0.22 | 2.78 ± 0.6 | |
50% reduction in the rate constant upon decreasing the pH from 8 to 6 in the presence of 1HP. In the absence of organic phosphates, the recombinant Hb still remains biphasic with a monotonic decrease in the rate constant as the pH is lowered. The fast phase remains between 10 and 15 times faster than native Hb with rate constants similar to liganded Hb. The slow phase is similar to native Hb at pH 8.0; however, both the rate constants of the recombinant Hb are not as sensitive to the drop in pH as in native Hb.

Ligand Binding Properties of Isolated Chains—With the observed alterations in functional properties of the recombinant Hb, we investigated whether the isolated chains had ligand binding properties similar to isolated native chains. Representative observed time courses for the recombinant of CO to isolated recombinant β^−αn and α−βn chains are shown in Fig. 4. The curves of both the native and recombinant α and β chains appear monophasic and can be fit to a single exponential. The dependence of the observed rates on ligand concentrations was examined using the equation:

\[ k_{\text{obs}} = k_{\text{on}} [\text{CO}] + k_{\text{CO}} \]  

(Eq. 1)

where \( k_{\text{on}} \) is the bimolecular second order association rate constant and \( k_{\text{CO}} \) is the dissociation rate constant for CO. The resultant pseudo first order rate constants determined from the fit depend linearly upon the amount of carbon monoxide present. The CO on rate, \( k_{\text{on}} \), of the recombinant α and native α chains were found to be identical within experimental error (Table III). Similarly the CO association rate, \( k_{\text{on}} \), of recombinant β chain is also linearly dependent upon the ligand concentration and the calculated rate constants are identical to that of native β-globin.

The dissociation rate constants for both native and recombinant α and β chains were determined by ligand displacement reaction with NO. In these experiments the protein-CO complex was mixed with a solution of NO which exhibits a higher affinity and a smaller dissociation rate constant than does CO. The concentration of NO, the replacing ligand, is high, and the observed rate is described by:

\[ k_{\text{obs}} = k_{\text{on}} + (k_{\text{CO}} [\text{CO}] / k_{\text{NO}}) \]  

(Eq. 2)

where \( k_{\text{CO}} \) and \( k_{\text{CO}} \) are the association and dissociation rate constant for the carbon monoxide and \( k_{\text{NO}} \) is the association rate constant of NO. For dissociation of CO from the α and β complexes, 1 atm of NO gas was mixed with the protein. Since \( k_{\text{NO}} \gg k_{\text{CO}} \) for all heme proteins (29) at high concentrations of NO the observed replacement rate constant determined is directly equal to the dissociation rate constant of NO (\( k_{\text{CO}} [\text{CO}] / k_{\text{NO}} \)) (Eq. 2). Under the conditions used, the observed rate approximates the off rate of CO. The rates determined are listed in Table III. The off rate determined for comparison of the chains indicate that there is little difference between either the recombinant α or β chains and the native α or β chains. Equilibrium constants determined by \( k_{\text{on}} / k_{\text{off}} \) confirm that the recombinant chains are similar to native chains.

Functional Properties of the Reassembled Recombinant Tetramer—The recombinant Hb was reassembled to determine whether the altered functional characteristics of the recombinant Hb described previously were due to alterations within the quaternary structure of the tetramer or a misfolding of the individual chains. Fig. 5 represents a typical oxygen-binding isotherm of the native HbA and reassembled recombinant Hb. The oxygen affinity of the reassembled material is slightly lower than that of native Hb, but the cooperativity was increased from 2.0 to 2.5 as measured by the Hill coefficient. Table II lists the oxygen binding data in the presence and absence of heterotropic allosteric regulators. While the recombinant Hb isolated from E. coli shows altered DPG response and Bohr effect, the reassembled recombinant Hb has oxygen binding properties and cooperativity similar to native Hb (Table IV).

Since the reassembled recombinant tetrameric Hb appears to show oxygen binding and cooperativity similar to native Hb, the CO combination kinetics to the reassembled deoxy tetramer were investigated to determine if the high affinity R-state-like hemes had been reorganized into a more well behaved tetramer. Fig. 5 represents typical time course of CO combination to the reassembled recombinant tetramer and to native Hb. The curves of the reassembled tetramer were nearly monophasic and fit well to a single exponential. The traces show a substantial loss in high affinity hemes that was observed for recombinant Hb but still did not show true autocatalytic behavior. Table V lists the calculated rate constants as a
function of pH and organic phosphate. The rate constant of the recombinant reassembled Hb are quite similar to native Hb. The response to organic phosphate and protons suggest that this material is very similar to native Hb.

**DISCUSSION AND CONCLUSION**

Recombinant Hb—The object of this study was to determine whether a recombinant tetrameric Hb produced completely in E. coli has physical and functional properties similar to native Hb. Initial characterization of the protein incorporated optical spectroscopy to probe the electronic environment of the porphyrin and electron paramagnetic resonance spectroscopy to investigate the ligation state of the heme iron. Both EPR and optical spectroscopy suggest that the formation of hexa coordinate recombinant Hb complexes are quite similar to that of native Hb.

While the physical characterizations of recombinant Hb are similar to native Hb, the O₂ binding properties are markedly different. Oxygen equilibrium binding properties of recombinant Hb provide an overall means to assess oxygen affinity, cooperativity and response to pH and organic phosphate. The Hill coefficient determined in this study is lower than other values reported for a similar assembled recombinant tetramer in E. coli (20) but the oxygen affinities are similar. Reduction of the Hill coefficient indicates destabilization of the tetramer possibly by either alteration of the dimer-tetramer equilibrium or perturbation within the ααββ₂ interface. Stabilization of the tetramer and regulation of oxygen affinity by organic phosphates and protons at secondary sites is critical for Hb function, and altered alloster for recombinant Hb may be indicative of small but significant structural changes. Since organic phosphates such as IHP and DPG both bind at the β1 N terminus (30, 31) and are critical for allosteric function, these effectors may be sensitive to perturbations due to the valine to methionine substitutions or alterations which perturb the binding site. Since the substitution of βVal → Met does not affect the allosteric regulation by organic phosphate, the observed alterations in organic phosphate binding of the completely recombinant Hb could imply changes within the central cavity of the recombinant Hb. Interaction with IHP suggests that these perturbations or rearrangements do not appear to sterically hinder the binding of organic phosphate but may affect the electrostatic interactions. The alterations in electrostatic interaction may possibly explain the reduction in DPG stabilization, while IHP stabilization is apparently unaffected. In addition significant reduction in the Bohr effect of the recombinant Hb strongly suggests that structural rearrangements may have occurred during in vivo assembly resulting in a change in the environment of the Bohr groups.

CO combination kinetics of deoxy Hb suggest that the deoxy recombinant Hb exists as a heterogenous population of at least two different reactive hemes. The slow phase of the recombinant Hb has a rate constant similar to native Hb suggesting one population may be a reasonably well behaved deoxy or T-state tetramer. The fast kinetic phase of the recombinant Hb may be a heterogeneous population of species which are unable to form a well behaved deoxy tetramer. The second order rate constant for the fast component is approximately a factor of 10 greater than that of a native Hb deoxy tetramer, but within a factor of 2–3 for combination of CO to R-state Hb (32) or to isolated chains (21, 33).

AllostERIC effectors were applied in the kinetic analysis to ascertain whether the biphasic behavior of the recombinant Hb was kinetically sensitive to IHP and pH. CO combination to deoxy recombinant Hb shows no change in the shape of the traces or the return to autocatalytic behavior upon addition of IHP or a drop in pH. The fast phase is sensitive to allosteric effects suggesting that this population is in a tetrameric form since dimers and isolated chains are not sensitive to IHP effects.

**TABLE V**

| pH       | HbA   | Reassembled recombinant Hb |
|----------|-------|-----------------------------|
| 6        | 1.3 ± 0.1 | 1.4 ± 0.1                  |
| 7        | 1.4 ± 0.1 | 1.5 ± 0.2                  |
| 7 + IHP  | 0.6 ± 0.1 | 0.5 ± 0.3                  |
| 8        | 3.0 ± 0.6 | 1.7 ± 0.1                  |

Experimental conditions were 50 mM Tris or Bis-Tris [Cl⁻] = 0.1 M at 20°C.
Reassembled Recombinant Tetramer—As discussed above the integrity of the heme binding pocket of the isolated α and β subunits appears to be retained, but the ability of the subunits to self-assemble into a well-behaved tetrameric Hb remains to be determined. To address this question, the isolated chains were reassembled and the recombinant tetramer was purified. It was found that in the absence of allosteric effectors, the reassembled recombinant Hb had a slightly lower affinity for oxygen and a similar Hill coefficient when compared to native Hb, but the cooperativity has increased from 2.0 to 2.5 when compared to recombinant Hb isolated from E. coli. This increase in cooperativity by reassembling the tetramer implies that some rearrangement of the quaternary structure may have occurred during reassembly which increased the stabilization of the tetramer.

An entirely different picture emerges when one compares the oxygen binding properties of the reassembled tetramer versus unmodified recombinant Hb in the presence of heterotrophic allosteric effectors. IHP effects of the reassembled recombinant Hb are similar to native HbA as would be expected because of the high affinity of IHP for the deoxy tetramer. In contrast the specific effect of DPG on the reassembled tetramer indicates a profound change to a lower oxygen affinity identical to that of native Hb. That the reassembled recombinant tetrameric Hb is affected by DPG may imply a rearrangement of the amino acid side chains within the central cavity that results in tighter binding of DPG and therefore an increase in stabilization of the tetramer. The increase in the Bohr effect of the reassembled tetramer to a more native-like behavior again implies a possible rearrangement of the reassembled recombinant Hb. The return of regulation by DPG and increase in the Bohr effect of the reassembled tetramer again argues against the substitution of Val → Met as the cause of the reduced heterotropic effect observed for the recombinant Hb, but rather provides support that the large side chain at this position is relatively innocuous to the heterotropic regulation of Hb and suggests a misassembly of the tetramer expressed in E. coli.

The kinetics of CO combination to deoxy Hb probe both the quaternary structure and the heme binding site of Hb. The results of the experiments from the reassembled recombinant Hb show a monophasic time course and fit well to a single exponential. This monophasic behavior did not change under varying conditions. Response of the reassembled tetramer to allosteric regulation by organic phosphates and protons provides an additional means to kinetically probe the functional behavior of the reassembled tetramer. The reassembled tetramer has a 60% reduction in the rate of CO bonding upon addition of IHP while the native has a 55% reduction in the rate. In addition the reassembled tetramer has a reduction in the rate with lowering of pH similar to that of native Hb. The appearance of monophasic behavior and the loss of the fast reacting component after reassembly of the tetramer further suggests that a rearrangement of the quaternary structure of the tetramer has occurred during the reassembly to produce a more native-like protein capable of carrying out full functional behavior.

Expression of recombinant Hb in E. coli produces a protein with a reduced cooperativity, loss of most response to allosteric regulation and multiphasic kinetics. It is clear that reassembly of the tetramer significantly reduces the heterogeneity associated with the recombinant Hb produced in E. coli and tends to restore correct functional behavior including allosteric regulation. The apparent correct functional behavior of the chains suggests that the placement of the subunits in the quaternary structure of the recombinant Hb expressed in E. coli is altered thereby not having full functional behavior. By isolating the subunits, and reassembling the tetramer we have shown that a
Functional tetrameric Hb is produced. In vivo expression of Hb requires that both α and β chains be present (9) suggesting that template-assisted folding between the two chains is required for proper folding and assembly of the tetramer. During expression, misassembly could occur due to misalignment of the template. This misassembly, however, does not produce an unstable Hb but rather produces a soluble tetrameric Hb physically similar to but functionally distinct from that of native Hb. This is quite unique among oligomeric proteins which misassemble, since these proteins usually precipitate into inclusion bodies. It has been known that some oligomeric proteins lack the inherent ability to correctly assemble into a protein with biological function. In many cases proteins are expressed as insoluble aggregates in inclusion bodies, solubilized in urea, then renatured and reassembled to form an active protein. Molecular chaperons are required, in some instances, to assemble these proteins into the correct structure. For example, plant ferrodoxin-NADP + oxidoreductase has recently been expressed in the presence of GroEL and GroES to produce an assembled native peptide, which when expressed in a chaperon-deficient strain of E. coli the protein failed to assemble properly (39). The large and small subunits of ribulose-bisphosphate carboxylase/oxygenase have also been coexpressed in a soluble form in E. coli, but the specific activity is lower than expected, presumably due to the inability to form a completely assembled complex (40). Bacterial luciferase, an αβ heterodimer, has been produced by expressing the individual peptides separately in E. coli, and assembling the dimer after each of the individual subunits is purified (41). The heterodimer that formed by this procedure failed to assemble into an active heterodimer, suggesting that the subunits had folded into a catalytically incompetent structure. Activity of bacterial luciferase was restored by incubating the heterodimer in the presence of 5 M urea (42).

The recombinant Hb expressed in E. coli may exist with multiple quaternary structures or conformations which are not in equilibrium with each other, and the perturbations are most notable in the deoxy or T-state structure. The structures are presumably similar to the native structure but not completely identical and may exist in a local energy minimum. By isolating the subunits and reassembling the tetramer the quaternary structures are interconverted to a more native-like form. This study, however, was unable to distinguish between a misassembled quaternary structure, or misfolded subunits which lead to misassembly, or both. Thus reassembling the recombinant tetramer provides a mechanism to produce site-directed mutants for detailed structure-function studies using a coexpression system for recombinant Hb.

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