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Oxygen binding properties of human mutant hemoglobins synthesized in *Escherichia coli*

(expression vector/protein engineering/protein folding)

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ABSTRACT **Human β-globin was synthesized in *Escherichia coli* as a cleavable fusion protein, using the expression vector pLcIIFXβ-globin [Nagai, K. & Thøgersen, H. C. (1984) *Nature (London)* 309, 810–812]. The fusion protein cIIFXβ-globin was purified to homogeneity and cleaved at the junction by blood coagulation factor X_a; the authentic β-globin was liberated. β-globin was folded *in vitro* and reconstituted with heme and α subunits to form $\alpha_2\beta_2$ tetramers. The oxygen binding properties of reconstituted Hb are essentially the same as those of human native Hb. Two mutant Hbs (Hb Nymphéas [Cys-93β→Ser] and Hb Daphne [Cys-93β→Ser, His-143β→Arg]) were constructed by site-directed mutagenesis using synthetic oligonucleotides. Hb Nymphéas showed a slightly increased oxygen affinity and diminished cooperativity with normal 2,3-diphosphoglyceric acid and slightly reduced alkaline Bohr effects. Hb Daphne showed low cooperativity with high oxygen affinity. The alkaline Bohr effect was slightly reduced but the diphosphoglycerate effect was enhanced by 50% by the His-143β→Arg mutation. As arginine is fully charged at physiological pH and has a long flexible side chain, diphosphoglycerate binds more strongly to Hb Daphne.**

Expression of cloned genes in appropriate host cells has made it possible to study protein functions by site-directed mutagenesis (1–5). Studies of hemoglobin (Hb) by this method are of particular interest since it is the only allosteric protein whose structures have been solved to atomic resolution in both the T (tight) and R (relaxed) states (6, 7). Subunit interaction, binding of various allosteric effectors, and interaction of oxygen with residues in the heme pocket can be studied in detail by introducing various mutations. The effects of such mutations on the electronic state of the heme and the mode of ligand binding can be studied spectroscopically (8).

We have developed a cleavable fusion protein expression vector to produce β-globin in *Escherichia coli* in amounts sufficient for biochemical and x-ray crystallographic studies (9). This method has several advantages over conventional ones. Because the β-globin coding sequence is joined to a short coding sequence of the λ *c*II gene, translation of the mRNA is initiated at the *c*II gene with extremely high efficiency. The sequence encoding the tetrapeptide Ile-Glu-Gly-Arg was inserted at the junction between the *c*II and the β-globin sequences. This is the tetrapeptide that precedes two cleavage sites for blood coagulation factor X_a in prothrombin (10). As a result, the fusion protein, cIIFXβ-globin, is cleaved by factor X_a only at the peptide bond following the tetrapeptide, and the authentic β-globin is liberated (9). In most eukaryotic proteins produced in *E. coli* by conventional methods, an extra methionine residue arising from the initiation codon remains attached to the amino terminus (12). Our fusion protein expression/cleavage system eliminates this residue. This is important for functional studies of β-globin since its amino terminus binds various allosteric effectors such as 2,3-diphosphoglyceric acid, inositol pentaphosphate, and adenosinetriphosphate (12); an extra residue at the amino terminus would change the oxygen binding properties of Hb significantly.

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MATERIALS AND METHODS

β-Globin Expression Vector. pLcFXβ-globin (9) was modified to carry out the large scale fermentation under category 0 conditions as defined by the Genetic Manipulation Advisory Group of the Medical Research Council. As shown in Fig. 1, the *Bgl* I–*Hin*dIII fragment of pLcIIFXβ-globin containing the origin of replication was replaced with the *Bgl* I–*Hin*dIII fragment of pUC9 (13) to form pLcIIFXβ-globin(nic$^-$) so that the plasmid had lost the nic site (the origin for conjugal transfer) and was no longer transmissible. QY13 (F^- lac_{am} trp_{am} *B B' bio-256* N^+ *cI857* Δ*H Sm*^{r} *recA*) was used as a host strain (a gift from S. Brenner).

Site-Directed Mutagenesis of β-Globin Gene. The *Sac* I–*Hin*dIII fragment of pLcIIFXβ-globin containing the β-globin cDNA sequence was cut out and inserted into the *Sac* I–*Hin*dIII site of phage M13 mp10 DNA to form M13 mp10 cIIFXβ-globin. Two mutagenic primers [KN20: d(GCTTGTCAGAGTGCAGC) and KN21: d(GATACTTGCGGGCTAGG)] were synthesized by using the phosphotriester method. The mutagenesis experiment was carried out as described by Carter *et al.* (14). The *Sac* I–*Hin*dIII fragment of M13 mp10 cIIFXβ-globin was inserted into pLmp10 to form pLcIIFXβ-globin(nic$^-$) with mutated sequences.

Protein Preparation. QY13 harboring pLcIIFXβ-globin(nic$^-$) was grown at 30°C in 2× TY medium (1 × 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) in the presence of ampicillin (25 μg/ml). When the optical density (600 nm) of the culture reached 1.0, the temperature was quickly raised and maintained at 42°C for 15 min, followed by further incubation at 37°C for 3–4 hr. The cells were harvested and frozen in liquid nitrogen. The cells (100 g) were thawed and suspended in 80 ml of 50 mM Tris·HCl (pH 8.0)/25% sucrose (wt/vol)/1 mM EDTA and lysed by addition of lysozyme (200 mg). Then, $MgCl_2$, $MnCl_2$, and DNase I were added to final concentrations of 10 mM, 1 mM, and 10 μg/ml, respectively. After a 30-min incubation 200 ml of 0.2 M NaCl/1% deoxycholic acid/1.6% Nonidet P-40 (vol/vol)/20 mM Tris·HCl (pH 7.5)/2 mM EDTA was added to the lysate, which was then centrifuged at 5000 × *g* for 10 min. Then, the pellet was suspended in 0.5% Triton X-100/1 mM EDTA and centrifuged. This procedure was repeated until a tight pellet was obtained (15). The protein pellet was finally dissolved in 8 M urea/25 mM Tris·HOAc (pH 5.0)/1 mM EDTA/1 mM dithiothreitol and applied to a 4 × 10 cm CM-Sepharose (Pharmacia) column equilibrated with the same buffer. The fusion protein cIIFXβ-globin was eluted

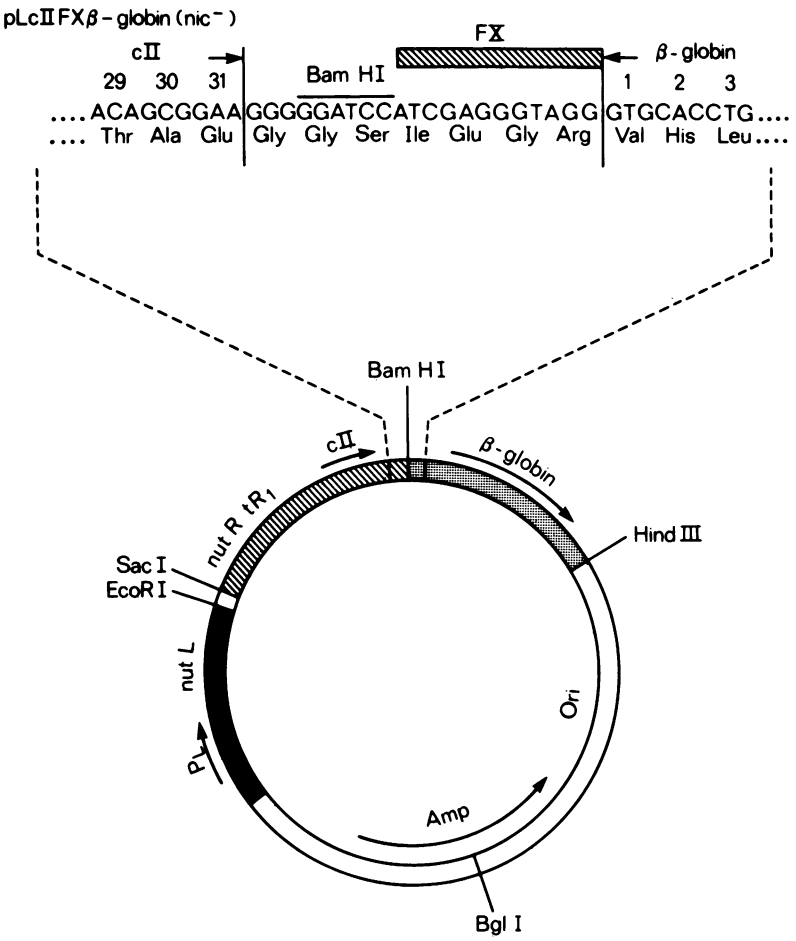

FIG. 1. Cleavable fusion protein expression vector, pLcIIFXβ-globin(nic⁻). The plasmid directs synthesis of a fusion protein consisting of the 31 amino-terminal residues of the λ *c*II protein, the Ile-Glu-Gly-Arg tetrapeptide, and the complete β-globin sequence under the control of λ P_L promoter. The *Hin*dIII–*Bgl* I fragment of pLcIIFXβ-globin (9) was replaced with the *Hin*dIII–*Bgl* I fragment of pUC 9 to remove the nic site.

with a linear gradient formed with 500 ml of 8 M urea/25 mM Tris·(HOAc (pH 5.0)/1 mM EDTA/1 mM dithiothreitol and 500 ml of the same buffer with 0.2 M NaCl. The fusion protein was further purified on a 5 × 600 cm Sephacryl S-200 (Pharmacia) column equilibrated with 5 M guanidine·HCl/50 mM Tris·HCl/1 mM EDTA/1 mM dithiothreitol to remove any trace of impurities which might prevent the folding of β-globin. The combined fraction, containing about 160 mg of cIIFXβ-globin, was extensively dialyzed against 50 mM Tris·HCl (pH 8.0)/100 mM NaCl and digested with 5 mg of blood coagulation factor X_a that had been activated with Russell's viper venom immobilized on cyanogen bromide-activated Sepharose-6B (a gift from H. C. Thøgersen). The cleaved fusion protein was extensively dialyzed against water and lyophilized. The protein was dissolved at a concentration of 5 mg/ml in 8 M urea/50 mM Tris·HCl (pH 8.0)/1 mM dithiothreitol, and the β-globin was folded by dilution to 0.3 mg/ml and was reconstituted with cyanoheme and α-chain in 1.2 molar excess. The reconstituted Hb was reduced with sodium dithionite under an atmosphere of CO in the presence of catalase (1 μg/ml) and purified by CM-cellulose chromatography as described (16).

Measurement of Oxygen Equilibrium Curves. The purified Hbs were concentrated and gel-filtered on a Sephadex G-25 (fine) (Pharmacia) column equilibrated with 1 mM Tris·HCl (pH 8.0). CO was removed from Hb by photolysis under a stream of oxygen. The oxygen equilibrium curves were determined with a continuous recording method (17). The concentration of metHb was determined spectroscopically before and after each measurement. The concentration of metHb was less than 10% after the measurement except for *E. coli* Hb A at pH 5.9, which was 12%.

RESULTS

Site-Directed Mutagenesis Using Oligonucleotides. Mutations were introduced into the β-globin cDNA sequence in M13 mp10 cIIFXβ-globin using mutagenic oligonucleotide primers. KN20 [d(GCTTGTCAGAGTGCAGC)] was used to replace Cys-93β d(TGT) with Ser d(TCT). About 30% of the plaques were positive by filter hybridization with the mutagenic primer which had been phosphorylated with [γ-^{32}P]ATP. Fig. 2 shows the DNA sequences of the Ser-93β mutant and the wild type (Cys-93β) in the mutated region. M13 mp10 cIIFXβ-globin(Ser-93β) was used to introduce the

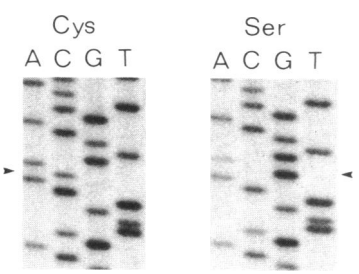

FIG. 2. Autoradiogram of the DNA sequencing gel. DNA sequences of the noncoding strand are shown for Hb Nymphéas (Ser-93β) and the wild type (Cys-93β).

second mutation, His-143β→Arg [d(CAC)→d(CGC)], with KN21 [d(GATACTTGCGGGCTAGG)]. The entire coding sequences of M13 mp10 cIIFXβ-globin(Ser-93β) and M13 mp10 cIIFXβ-globin(Ser-93β, Arg-143β) were determined after plaque purification to ensure that there were no other mutations. The replicative forms of these clones were prepared and cleaved with *Sac* I and *Hin*dIII. The *Sac* I–*Hin*dIII fragment was inserted into pLmp10 to form pLcIIFXβ-globin with mutated β-globin sequences.

Characterization of Hb Reconstituted with β-Globin Synthesized in *E. coli*. Fig. 3 shows $NaDodSO_4$/polyacrylamide gel analyses of Hb at various stages of purification (9). The cIIFXβ-globin fusion protein represents about 5–10% of the total cellular protein. The first pellet contained some membrane proteins but, after the Triton X-100 washing step, cIIFXβ-globin was more than 90% pure and essentially free from the membrane fraction. The fusion protein was purified by two chromatographic steps to remove any trace of impurities that might prevent folding. Wild type and the Cys-93β→Ser mutant of cIIFXβ-globin were cleaved by coagulation factor X_a only at the peptide bond following the Ile-Glu-Gly-Arg sequence. No further cleavages took place even after prolonged incubation. On the other hand, the His-143β→Arg mutant was also cleaved at the Arg-Lys bond between residues 143 and 144 in β-globin, and the reaction was stopped at 90% completion by addition of 1 mM dithiothreitol.

When the reconstituted Hb A and Hb Nymphéas were purified on a CM-52 column, two peaks were eluted: one is the reconstituted tetramer, and the second is the excess α-chain. Hb Daphne gave an extra peak which corresponds to des His-Tyr-Lys Hb in which three carboxyl-terminal residues, 146, 145, and 144, have been removed from β-globin by factor X_a.

All reconstituted Hbs showed optical absorption spectra indistinguishable from those of native human Hb A. Isoelectric focusing gel analysis showed that Hb A (*E. coli*) and Hb Nymphéas had the same isoelectric point as native human Hb A (pI = 6.95). The isoelectric point of Hb Daphne (pI = 7.32) was higher than that of Hb A due to the His→Arg replacement.

Titration of sulfhydryl group with *p*-hydroxymercuribenzoate showed that there were no reactive sulfhydryl groups in Hb Nymphéas and Hb Daphne because Cys-93β had been replaced with Ser.

Oxygen Binding Properties of Mutant Hbs Synthesized in *E. coli*. The oxygen binding properties of Hbs produced in *E. coli* are shown in Fig. 4 and Table 1 together with those of human Hb A. The P_{50} is the partial oxygen pressure required to give 50% saturation of Hb. The Hill constant n_{max} is the maximal slope of the Hill plot and is a measure of the cooperativity (18). The effect of pH on oxygen affinity,

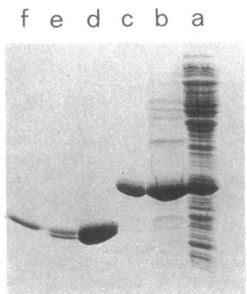

FIG. 3. $NaDodSO_4$/polyacrylamide gel analysis of the cleavable fusion protein cIIFXβ-globin at various stages of purification. Lanes: a, total cellular protein; b, pellet after washing with Triton X-100 solution; c, purified cIIFXβ-globin; d and e, reconstituted Hb; f, human β-globin.

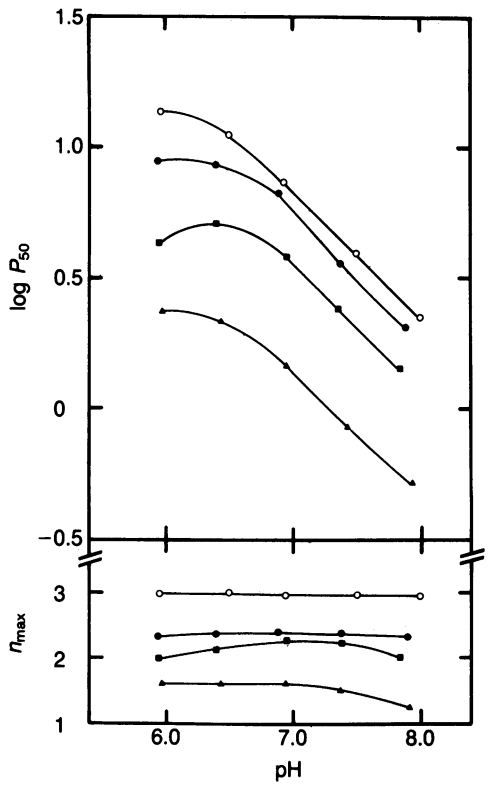

FIG. 4. Oxygen binding properties of wild-type and mutant Hbs synthesized in *E. coli*. ○, Control human Hb A; ●, Hb A reconstituted with *E. coli* β-globin; ■, Hb Nymphéas; ▲, Hb Daphne. Experimental conditions: buffer 0.05 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bistris), 0.05 M Tris/100 mM HCl; temperature 25°C; heme concentration, 30 μM. P_{50} is the partial pressure of oxygen in torr (1 torr = 133 Pa) at 50% saturation of Hb, and n_{max} is the maximal slope of the Hill plot.

known as the Bohr effect, is given by $\Delta \log P_{50}/\Delta pH$, and the diphosphoglycerate (DPG) effect by $\log P_{50}^{DPG} - \log P_{50}^{str}$, where P_{50}^{DPG} and P_{50}^{str} are partial oxygen pressures at 50% saturation in the presence and absence (str indicates stripped) of diphosphoglycerate. At pH >7, Hb A (*E. coli*) had a slightly increased oxygen affinity and reduced cooperativity compared to human Hb A. At pH <7, the oxygen affinity increased due to auto-oxidation of *E. coli* Hb, which was as much as 12% after the measurement. Between pH 7 and 8, the alkaline Bohr effect and the diphosphoglycerate effects were identical in *E. coli* and human Hb A (Table 1).

Hb Nymphéas (Cys-93β→Ser) showed only slightly increased oxygen affinity and reduced cooperativity, a slightly reduced alkaline Bohr effect, and a normal diphosphoglycerate effect, but Hb Daphne showed a high oxygen affinity and reduced cooperativity, implying that the T state is destabi-

Table 1. Heterotropic effects of oxygen binding

Hemoglobin	Alkaline Bohr effect, $\Delta \log P_{50}/\Delta pH$	Diphosphoglycerate effect, $\log P_{50}^{DPG} - \log P_{50}^{str}$
Control human Hb	−0.500	0.417
$\alpha_2\beta$(*E. coli*)$_2$	−0.505	0.382
Hb Nymphéas (Cys-93β→Ser)	−0.474	0.425
Hb Daphne (Cys-93β→Ser, His-143β→Arg)	−0.458	0.646

Experimental conditions are as described for Fig. 3. The alkaline Bohr effect was measured between pH 7 and 8. The diphosphoglycerate effect was measured at pH 6.5 in the presence and absence of 1 mM diphosphoglycerate.

lized by the His-143β→Arg mutation. The alkaline Bohr effect was slightly decreased, and the diphosphoglycerate effect was increased by 50%. Arginine is a stronger base and has a longer, more flexible side chain than histidine and, therefore, binds diphosphoglycerate more strongly.

DISCUSSION

By using the cleavable fusion protein expression vector pLcIIFX(nic⁻), many eukaryotic proteins, including human β-globin, chicken β-actin, chicken myosin light chain (unpublished results), *Xenopus* histone H2A (M. Waye, personal communication), and human myoglobin (22), have been produced in amounts sufficient for biochemical and x-ray crystallographic studies (0.1–1 g). Since translation of mRNA is initiated at the λ *c*II gene, the mRNA is efficiently translated regardless of the gene joined downstream, and therefore, large amounts of eukaryotic proteins can be produced.

Blood coagulation factor X_a cleaved the wild type and the Cys-93β→Ser mutant of the cIIFXβ-globin fusion protein exclusively at the peptide bond following the tetrapeptide Ile-Glu-Gly-Arg even though the fusion protein contains 6 other arginine and 14 lysine residues. However, the cIIFXβ-globin (His-143β→Arg, Cys-93β→Ser) mutant was also cleaved slowly within the β-globin. In this mutant the Arg-143β residue introduced by the mutation is followed by Lys-144β. The rate of cleavage by trypsin-like proteases is generally enhanced if a lysine or arginine residue occupies the P_1' position.

Most eukaryotic proteins produced in *E. coli* by conventional methods have an extra methionine residue at the amino terminus that has arisen from the initiation codon. The cleavable fusion protein expression system has enabled us to produce eukaryotic proteins in *E. coli* with natural amino-terminal residues.

Most eukaryotic proteins produced in *E. coli* are insoluble in the bacterial cell and form inclusion bodies (11, 15) that can be dissolved only by strong denaturing reagents such as 8 M urea or 6 M guanidine·HCl. The proteins must then be purified by ion-exchange chromatography in 8 M urea, where their α-amino groups are readily carbamoylated by the cyanate formed there. The fusion protein cleavage method minimizes the risk of carbamoylation since the amino-terminal amino acid is protected by the extra amino-terminal peptide. The normal diphosphoglycerate effect of Hb Nymphéas proves that the α-amino group of the β-chain has not been carbamoylated (19).

Cys-93β is conserved in all mammalian Hbs but is replaced by serine in some fish Hbs, which exhibit the Root effect. This consists of a drastic lowering of the oxygen affinity and cooperativity as the pH drops from 7 to 6. Perutz and Brunori (20) proposed that the replacement of Cys-93β by serine might explain the Root effect, because the serine hydroxyl would make strong hydrogen bonds with the carboxyl-terminal histidine, which would stabilize its salt bridge with Asp-94β in the quaternary deoxy structure at acid pH. Our results show that the single replacement Cys-93β→Ser is not sufficient to generate these bonds in human deoxyhemoglobin and that additional replacements may be necessary to bring the serine and the histidine together.

The replacement His-143β→Arg was chosen because it would halve the acid Bohr effect, which is absent from teleost fish Hbs, and also because it lowers cooperativity at high pH, which is a property of these fish Hbs. Observations on hemoglobins Abruzzo (His-143β→Arg) (21) and Hb Daphne (Cys-93β→Ser, His-143β→Arg) show that this replacement does have that effect because the extra positive charge in the central cavity destabilizes the T structure. The substitution also enhances the interaction with diphosphoglycerate.

We have shown that active Hb can be reconstituted from human β-globin produced in *E. coli*; therefore, any mutant Hb can now be prepared by site-directed mutagenesis. Our method should provide a useful tool for studying the mechanism of allosteric control, folding, and molecular evolution of protein molecules.

Note Added in Proof. Deoxy Hb Nymphéas has been crystallized, and x-ray diffraction data has been collected to 2.8 Å. A difference Fourier of deoxy Hb (Nymphéas minus A) shows a strong negative peak on the sulfur of Cys-93β and weaker pairs of positive and negative density nearby. There is no significant density elsewhere, which confirms that there is no other difference between the β chain made in *E. coli* and the native human one. The map shows that the hydroxyl group of Ser-93β forms a hydrogen bond with Asp-94β that is in equilibrium with the normal bond between Asp-94β and His-146β. Since this bond normally stabilizes the T structure and accounts for 40% of the alkaline Bohr effect, its partial disruption explains the increased oxygen affinity and reduced Bohr effect of Hb Nymphéas (unpublished result).

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