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# Studies of Haemoglobin Functions by Site-Directed Mutagenesis

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# Studies of haemoglobin functions by site-directed mutagenesis

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Human β-globin was synthesized in *Escherichia coli* as a cleavable fusion protein by using the expression vector pLcIIFXβ-globin(nic$^-$). The authentic β-globin was liberated by digestion with blood coagulation factor $X_a$ and $\alpha_2\beta_2$ tetramers were reconstituted. The oxygen-binding properties of reconstituted haemoglobin (Hb) were essentially the same as those of human native Hb. Two mutant haemoglobins were constructed by site-directed mutagenesis. Hb Nymphéas (Cys-93β→Ser) showed a slightly increased oxygen affinity and diminished co-operativity with normal DPG (2,3-diphosphoglycerate) effect and slightly reduced alkaline Bohr effects. Hb Daphne (Cys-93β→Ser, His-143β→Arg) showed low co-operativity with high oxygen affinity. The alkaline Bohr effect was slightly reduced, but the DPG effect was enhanced by 50% by the His-143β→Arg mutation.

Recent advances in expression vectors have made it possible to prepare large amounts of proteins from manipulatable DNA sequences (Harris 1983; Neuberger *et al.* 1984). In combination with site-directed mutagenesis, it is now possible to study protein functions by introducing mutations at will (Winter *et al.* 1982). In this paper, we shall describe the functional properties of man-made haemoglobin (Hb) mutants.

Human and fish haemoglobins show distinct functional differences. The oxygen affinities of both haemoglobins are reduced by lowering the pH, but certain fish haemoglobins show a drastic lowering of the oxygen affinity and co-operativity as the pH drops below 7.0. This is known as the Root effect and is thought to enhance the discharge of oxygen to the swim bladder. The homology of amino-acid sequences between human and fish haemoglobins is only 40%, and therefore it is not easy to point to any single amino-acid residue as responsible for the Root effect. After comparing the amino-acid sequence and the functional properties of haemoglobins from different species, Perutz & Brunori (1982) proposed that a single mutation Cys-93β→Ser might be sufficient to produce the Root effect in human Hb.

By using the cleavable fusion protein expression vector (Nagai & Thøgersen 1984), we have produced two mutant haemoglobins: Hb Nymphéas (Cys-93β→Ser) and Hb Daphne (Cys-93β→Ser, His-143β→Arg) and have investigated the effect of these amino acid replacements on the oxygen-binding properties.

## Methods

### *Protein synthesis*

*E. coli* strain QY13, harbouring pLcIIFXβ-globin, was grown at 30 °C in 2 X TY medium (16 g of tryptone, 10 g of yeast extract and 5 g of NaCl per litre) with 25 μg ml$^{-1}$ ampicillin to an optical density (600 nm) of 1.0. Synthesis of the CIIFXβ-globin fusion protein was

induced by inactivation of the temperature-sensitive repressor ($cI_{857}$) at 42 °C. The cells were harvested after 3 h incubation at 37 °C and processed as described by Nagai *et al.* (1985). The CIIFXβ-globin fusion protein was purified by two chromatographic steps and digested with bovine blood coagulation factor $X_a$ to liberate the authentic β-globin (Nagai *et al.* 1985). The $\alpha_2\beta_2$ tetramets were reconstituted with β-globin which has been synthesized in *E. coli*.

### *Introduction of mutations in β-globin by site-directed mutagenesis*

The *Sac I-Hind III* fragment of pLcIIFXβ-globin was cloned into M13 mp10 to form M13 mp10 cIIFXβ-globin. Its single-stranded DNA was used as a template for site-directed mutagenesis. Two mutagenic primers, KN20: dGCTTGTCAGAGTGCAGC and KN21: dGATACTTGCGGGCTAGG were used to introduce the Cys-93β → Ser and His-143β → Arg mutations. The mutagenesis experiments were performed by using mutL strain as described by Carter *et al.* (1984). The mutated β-globin sequences were cloned back into pLcIIFXβ-globin(nic$^-$).

### *Determination of oxygen equilibrium curves of haemoglobins*

The reconstituted carbomonoxy haemoglobins were gel-filtered against 1 mM Tris–HCl, pH 8.0 and converted to the oxy form by photolysis under a stream of oxygen. The oxygen equilibrium curves were determined with a continuous recording method (Rochette *et al.* 1984). The concentration of metHb was found to be less than 10% after the measurement, except for *E. coli* Hb A at pH 5.9, which was 12%.

## Results

### *Protein expression vector*

As shown in figure 1, pLCIIFXβ-globin directs the synthesis of a fusion protein consisting of the N-terminal 31 amino-acid residues of the λ cII protein, the Ile–Glu–Gly–Arg tetrapeptide and human β-globin under the control of the λ $P_L$ promoter. Because translation of the mRNA is initiated at the λ cII gene with extremely high efficiency, the CIIFXβ-globin fusion protein is synthesized in high yield. The fusion protein, which represents about 5–10% of the total cellular protein, was purified to homogeneity by two chromatographic steps.

### *Cleavage of the fusion protein by blood coagulation factor $X_a$*

We have inserted the Ile–Glu–Gly–Arg tetrapeptide sequence between the λ cII and β-globin sequences in the CIIFXβ-globin fusion protein. This tetrapeptide precedes two cleavage sites for factor $X_a$ in prothrombin (Magnusson *et al.* 1975). The CIIFXβ-globin was cleaved by factor $X_a$ at the single peptide bond after the Ile–Glu–Gly–Arg tetrapeptide and the authentic β-globin was liberated (Nagai & Thøgersen 1984).

Most eukaryotic proteins produced in *E. coli* by conventional methods have an extra Met residue at the N-terminus which has arisen from the initiation codon (Harris 1983), but this is eliminated by the cleavable fusion-protein expression system. This is important for functional studies of β-globin because its N-terminus binds allosteric effectors such as 2,3-diphosphoglycerate (Arnone 1972).




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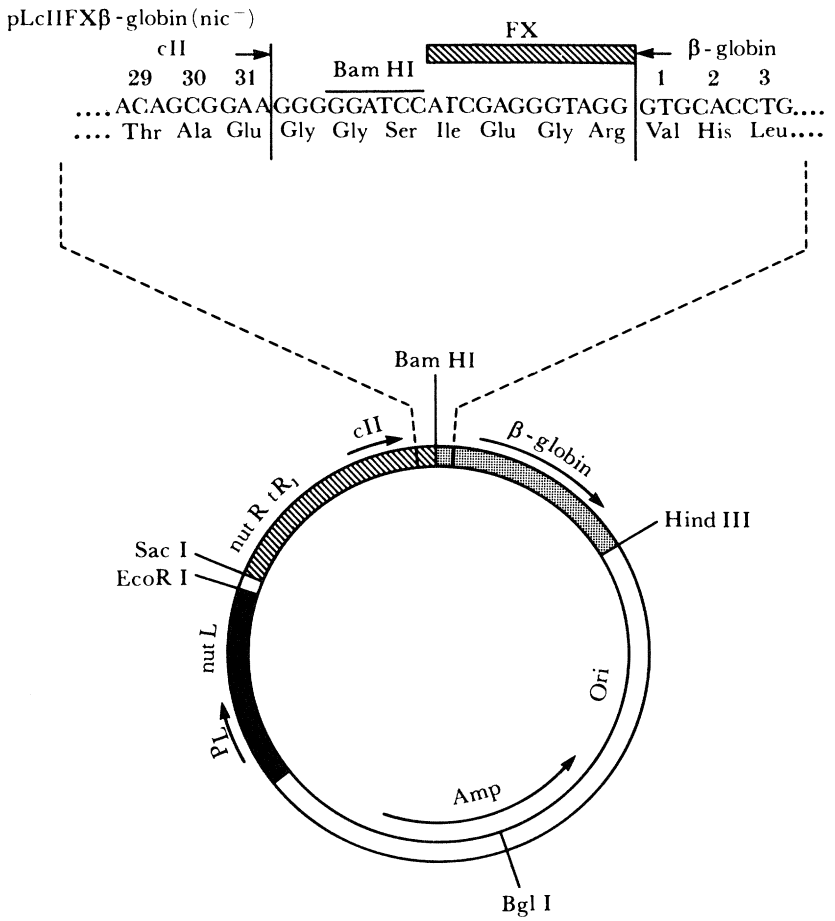


FIGURE 1. Cleavable fusion-protein expression vector, pLcIIFXβ-globin(nic⁻). The plasmid directs synthesis of a fusion protein consisting of the 31 N-terminal residues of the λ cII protein, the Ile–Glu–Gly–Arg tetrapeptide and the complete β-globin sequence under the control of λ $P_L$ promoter. The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

*Oxygen-binding properties of mutant haemoglobins synthesized in E. coli*

Figure 2 shows the oxygen-binding parameters of the wild-type and of two mutant haemoglobins produced in *E. coli*. $P_{50}$ is the partial pressure of oxygen at 50% saturation of Hb. The Hill coefficient $n_{max}$ is the maximal slope of the Hill plot and is a measure of the co-operativity (Baldwin 1975). The oxygen-binding properties of Hb A (*E. coli*) are essentially the same as those of native human Hb.

The Cys-93β→Ser mutation in Hb Nymphéas (Cys-93β→Ser) slightly increased the oxygen affinity and reduced the co-operativity. As shown in table 1, Hb Nymphéas had a slightly reduced alkaline Bohr effect, but its DPG effect was normal. Hb Daphne (Cys-93β→Ser, His-143β→Arg) showed a high oxygen affinity and reduced co-operativity, implying that the T state was destabilized by the His-143β→Arg mutation. The alkaline Bohr effect was slightly decreased, but the DPG effect was increased by 50% by the His-143β→Arg mutation.

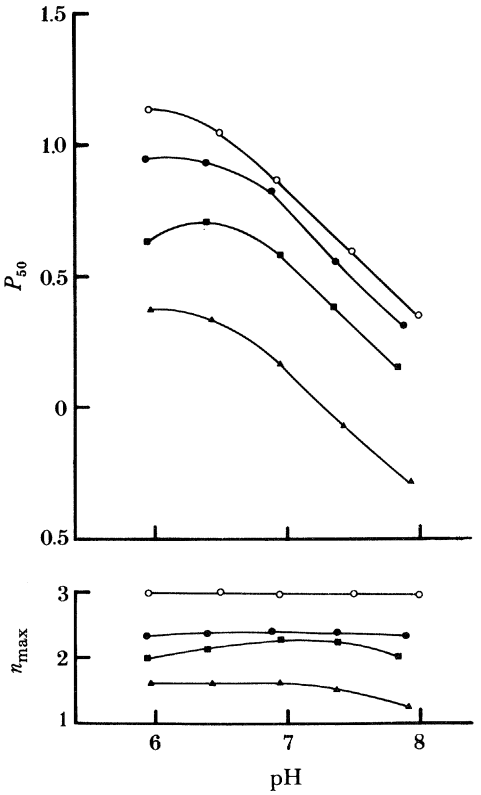


FIGURE 2. Oxygen-binding properties of wild-type and mutant Hbs synthesized in *E. coli*: ○, control human Hb A; ●, Hb A reconstituted with *E. coli* β-globin; ■, Hb Nymphéas; ▲, Hb Daphne. Experimental conditions: 0.05 M bis-Tris; 0.05 M Tris; 100 mM $Cl^-$ at 25 °C; haem concentration 30 μM. $P_{50}$ is the partial pressure of oxygen (in torr) at 50 % saturation of Hb, $n_{max}$ is the maximal slope of the Hill plot.

TABLE 1. HETEROTROPIC EFFECTS OF OXYGEN BINDING

| haemoglobin | alkaline Bohr effect ($\Delta \lg P_{50}/\Delta$pH) | DPG effect ($\lg P_{50}^{DPG} - \lg P_{50}^{str}$) |
|---|---|---|
| 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 in figure 2. The alkaline Bohr effect was measured between pH 7 and 8. The DPG effect was measured at pH 6.5 in the presence of 1 mM DPG. $P_{50}^{DPG}$ is the partial pressure of oxygen at 50 % saturation in the presence of 1 mM DPG. $P_{50}^{str}$ is the partial pressure of oxygen at 50 % saturation of stripped haemoglobin.

## DISCUSSION

By using the cleavable fusion protein expression vector, the authentic human β-globin was obtained from *E. coli* extract in amounts sufficient for biochemical and X-ray crystallographic studies. Hb A was reconstituted with β-globin synthesized in *E. coli* and its oxygen-binding properties were found to be essentially the same as those of native human Hb A. Therefore, any mutant Hb can now be prepared by site-directed mutagenesis.

We have studied the functional properties of two mutant haemoglobins: Hb Nymphéas (Cys-93β→Ser) and Hb Daphne (Cys-93β→Ser, His-143β→Arg). Cys-93β is conserved in

all mammalian Hb but replaced by Ser or Ala in fish and amphibians (Perutz & Brunori 1982). The haemoglobins of teleost fish exhibit the Root effect, which consists of a drastic lowering of the oxygen affinity and co-operativity at acidic pH. These haemoglobins have Ser in position 93β. Perutz & Brunori (1982) proposed that the replacement of Cys-93β→Ser might explain the Root effect because the serine hydroxyl group would make strong hydrogen bonds with the C-terminal carboxyl group of the β-chain. This bond would stabilize the quaternary T structure at acidic pH. The oxygen-binding properties of Hb Nymphéas show that the single mutation Cys-93β→Ser is not sufficient to generate the Root effect in human Hb.

Another property of teleost fish haemoglobins is the lowering of the co-operativity at alkaline pH. We suspected this to be a result of the substitution His-143β→Arg, which is also present in the natural human mutant Hb Abruzzo. We therefore prepared the double mutant Hb Daphne. Hb Abruzzo (His-143β→Arg) (Bonaventura *et al.* 1975) and Hb Daphne (Cys-93β→Ser, His-143β→Arg) do show a high oxygen affinity and low co-operativity at alkaline pH because the additional positive charge in the central cavity destabilizes the T structure. The substitution His-143β→Arg enhances the interaction with DPG and thus both Hb Daphne and Hb Abruzzo show an increased DPG effect.

Now we can study the effect of any mutation on functional properties of Hb. This should provide a useful tool for studying the mechanism of allosteric control, folding, and molecular evolution of Hb.

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## References

Arnone, A. 1972 X-ray diffraction study of binding of 2,3-diphosphoglycerate to human deoxyhaemoglobin. *Nature, Lond.* **237**, 146–149.

Baldwin, J. M. 1975 Structure and function of haemoglobin. *Prog. Biophys. molec. Biol.* **29**, 227–320.

Bonaventura, C., Bonaventura, J., Amiconi, G., Tentori, L., Brunori, M. & Antonini, E. 1975 Hemoglobin Abruzzo [β143(H21) His-Arg]. *J. biol. Chem.* **250**, 6273–6277.

Carter, P. J., Winter, G., Wilkinson, A. J. & Fersht, A. R. 1984 The use of double mutants to detect structural changes in the active site of the tyrosyl-tRNA synthetase (*Bacillus stearothermophilus*). *Cell* **38**, 835–840.

Harris, T. J. R. 1983 Expression of eukaryotic genes in *E. coli*. In *Genetic engineering 4* (ed. R. Williamson), pp. 127–185. New York: Academic Press.

Magnusson, S., Petersen, T. E., Sottrup-Jensen, L. & Claeys, H. 1975 Complete primary structure of prothrombin: Isolation, structure and reactivity of ten carboxylated glutamic acid residues and regulation of prothrombin activation by thrombin. In *Protease and biological control* (ed. E. Reich, D. B. Rifkin & E. Shaw), pp. 123–149. New York: Cold Spring Harbor Laboratory.

Nagai, K., Perutz, M. F. & Poyart, C. 1985 Oxygen binding properties of human hemoglobin mutants synthesized in *Escherichia coli*. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7252–7255.

Nagai, K. & Thøgersen, H. C. 1984 Generation of β-globin by sequence-specific proteolysis of a hybrid protein produced in *Escherichia coli*. *Nature, Lond.* **309**, 810–812.

Neuberger, M. S., Williams, G. T. & Fox, R. O. 1984 Recombinant antibodies possessing novel effector functions. *Nature, Lond.* **312**, 604–608.

Perutz, M. F. & Brunori, M. 1982 Stereochemistry of cooperative effects in fish and amphibian haemoglobins. *Nature, Lond.* **299**, 421–426.

Rochette, J., Poyart, C., Varet, B. & Wajcman, H. 1984 A new hemoglobin variant altering the $\alpha_1\beta_2$ contact: Hb Chemilly $\alpha_2\beta_2$ 99(G1)Asp→Val. *FEBS Lett.* **166**, 8–12.

Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M. & Smith, M. 1982 Redesigning enzyme structure by site-directed mutagenesis: tyrosyl-tRNA synthetase and ATP binding. *Nature, Lond.* **299**, 756–758.





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