Additive Effects of β Chain Mutations in Low Oxygen Affinity Hemoglobin βF41Y,K66T*

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In order to decrease significantly the oxygen affinity of human hemoglobin, we have associated the mutation βF41Y with another point mutation also known to decrease the oxygen affinity of Hb. We have synthesized a recombinant Hb (rHb) with two mutations in the β chains: rHb βF41Y,K66T. In the absence of 2,3-diphosphoglycerate, additive effects of the mutations are evident, since the doubly mutated Hb exhibits a larger decrease in oxygen affinity than for the individual single mutations. In the presence of 2,3-diphosphoglycerate, the second mutation did not significantly increase the \( P_{50} \) value relative to the single mutations. However, the kinetics of CO binding still indicate combined effects on the allosteric equilibrium, as evidenced by more of the slow bimolecular phase characteristic of binding to the deoxy conformation.

Dimer-tetramer equilibrium studies indicate an increase in stability of the mutants relative to rHb A; the effects on the allosteric equilibrium, as evidenced by more of the slow bimolecular phase characteristic of binding to the deoxy conformation.

The search for human hemoglobin (Hb) variants exhibiting a low oxygen affinity without requiring 2,3-diphosphoglycerate (2,3-DPG) is of interest in the view of producing a blood substitute. With this objective, we have previously synthesized the recombinant Hb (rHb) βF41Y using the genetic engineering approach (1). The naturally occurring mutated Hb βF41Y, first described by Burkert et al. (2), is known as Hb Mequon. The mutation βF41Y occurs in an important region of the subunit interface, which undergoes large rearrangements in the transition between the deoxy (T state) and the liganded (R state) conformations (3). We have shown that the recombinant Hb βF41Y exhibits a lower oxygen affinity than Hb A, with a well preserved cooperativity of oxygen binding and without increasing the rate of autoxidation. The decreased oxygen affinity of rHb βF41Y is attributed mainly to an increase in the allosteric constant, \( L_o = T_1/R_0 \), because the oxygen equilibrium dissociation constants for the T and R states, \( K_T \) and \( K_R \), respectively, were not modified (1).

Based on the crystallographic structure of Hb A (4), it appears that there could be an additional hydrogen bond in the deoxy conformation between a tyrosyl residue at the β41(C7) site and the carbonyl of the β97(FG4) His residue within the same chain. Such an interaction, coupled with the native interchain hydrogen bond between the Tyr-α42(C7) and Asp-β69(G1) residues, would help stabilize the deoxy state of Hb βF41Y.

In addition to the amino acids involved in the \( \alpha_1 \beta_2 \) interface, other key residues are important in the cooperative ligand binding to human Hb. Specifically, the amino acids implicated in the heme contact have a crucial role. The study of naturally occurring human mutants has also confirmed the importance of these regions. Indeed, over 600 natural variants of Hb have been described (5), providing information about the role of certain residues in the structural changes between the deoxy and oxy conformations. Among these natural mutants, 54 displayed a decreased oxygen affinity, with 7 and 47 for the \( \alpha \) and \( \beta \) subunits, respectively. In particular, Hb Chico (βK66T) with a mutation close to the heme group exhibits a low oxygen affinity and a slight instability (6). In deoxy Hb A, the residue Lys-β66 (E10) forms a salt bridge with the carboxyl group of one propionic acid of the \( \beta \)-chain heme; this contact does not exist in the liganded form (4). X-ray analysis of Hb Chico has shown that Thr-β66 may form a hydrogen bond with His-β63 via a bridging water molecule. This introduces additional steric hindrance to ligand binding to the T state (7).

With a view to study the combined effects of the mutations F41Y and K66T in the same \( \beta \) subunit, we have produced an artificial human Hb βF41Y,K66T. We report here the functional properties of the double mutant compared with those of native Hb A and the singly mutated Hbs.

MATERIALS AND METHODS

The Lys-β66 → Thr mutation was introduced by site-directed mutagenesis into the \( \beta \)-globin cDNA containing the code for the mutation Phe-β41 → Tyr. The doubly mutated \( \beta \) globin was produced as a fusion protein in *Escherichia coli* using the expression vector pATprTet-cIIH-FX-βgb. After purification and cleavage of the fusion protein by digestion with bovine activated coagulation factor X, the \( \alpha_1 \beta_2 \) tetramer was reconstituted in the presence of cyanohemin and native carbonmonoxy \( \alpha \) subunits (8, 9). The structure of the mutated chain was checked by reversed-phase high performance liquid chromatography of tryptic digest and amino acid analysis of the two mutated peptides. The purity of the Hb was controlled by isoelectric focusing electrophoresis with a pH gradient ranging from 6.0 to 8.0. The heat stability was tested by incubating rHb βF41Y,K66T and Hb A (100 \( \mu \)g on a heme basis) at 65 °C in 10 mM phosphate buffer, pH 7.0 (10).

The tetramer-dimer equilibrium was studied by gel filtration on a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden) as described by Manning et al. (11). All experiments were performed at 25 °C, in 150 mM Tris acetate buffer, pH 7.5. For concentrations of Hb ranging from 2 to 500 \( \mu \)g on a heme basis, 10-\( \mu \)l aliquots were applied and eluted at a flow rate of 0.4 ml/min. The absorbance of the eluent was measured at 415 and 280 nm. Diaspirin cross-linked

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† The abbreviations used are: 2,3-DPG, 2,3-diphosphoglycerate; rHb, recombinant Hb.
(DLC) Hb was used as a control for undissociable tetrameric Hb, and the peak position of the dimer was determined using the dimeric natural mutant Hb Rothschild (12).

The rate of oxidation for liganded Hb samples was measured by absorption spectrophotometry (SLM-Aminco DW2000) at 37 °C for samples under 1 atm of oxygen or under air (13). Hb solutions were 40 μM in heme in 20 mM potassium phosphate at pH 7.0.

Oxygen equilibrium curves were recorded with a continuous method using the Hemox Analyzer system (TCS, Huntington Valley, PA) (14). The amount of metHb calculated from the visible absorption spectra was found to be less than 5% at the end of the recordings.

Fluorescence studies were performed on 10 μM Hb solutions (on a heme basis) in 10 mM phosphate buffer, pH 7.0, using an SLM 8000 spectrofluorometer. Emission spectra were recorded for both the buffer and the liganded Hb samples.

Kinetics of CO or oxygen recombination were obtained after flash photolysis using 10-ns YAG laser pulses (Quantel, France) providing 160 mJ at 532 nm (15). Measurements were made at 25 °C, 50 mM bis-Tris at pH 7.0, 100 mM NaCl, for samples equilibrated under air or 1 or 0.1 atm of CO. Kinetics were recorded at different laser energies to probe the Hb tetramer at different ligand saturation levels.

The CO dissociation rate was determined from the kinetics of replacement of CO by NO, with measurements of full spectra versus time using a diode array spectrophotometer (HP 8453).

Kinetics of O2 replacement by CO were measured with a stopped-flow apparatus (Biologic, France) with detection at 420 nm. Experimental conditions were 50 mM bis-Tris at pH 7.0, 100 mM NaCl, at 25 °C. The dead time of this apparatus with a cuvette of 1-cm optical path length is 2 ms. Hb samples equilibrated under 1 atm of oxygen were mixed with a solution equilibrated under 1 atm of CO containing the oxygen scavenger sodium dithionite. The final concentrations after mixing were 5 μM in heme, 5 mM sodium dithionite, 0.6 mM O2, and 0.5 mM CO.

RESULTS

Properties of rHb βF41Y,K66T—Analysis of the purified rHb βF41Y,K66T by isoelectric focusing showed that it migrated as a single band (pI = 6.4), with a more cathodic position relative to Hb A (pI = 6.98). Fluorescence studies did not show significant differences between the doubly mutated rHb and native Hb A; the highly quenched emission indicates a correctly recomposed (folded) rHb. The UV and visible absorption spectra of rHb βF41Y,K66T in carboxylated and oxygenated forms were identical to those of native Hb A. Notably, the ratios of absorbance intensity between the Soret band and UV peak at 280 nm were normal, 4.87 and 3.5 for the mutated HbCO and HbO2, respectively. The oxy form of rHb βF41Y,K66T exhibited the same fraction denaturation as Hb A after a 20-min incubation at 65 °C.

Tetramer-Dimer Equilibrium—Hb A, rHb A, rHb βF41Y, and rHb βF41Y,K66T in the liganded form were eluted as a single peak whose position varied between tetrameric and dimeric forms when the Hb concentrations were in the range of the dissociation constant K4,2 value. The K4,2 value for rHb A was 2-fold higher than that for natural Hb A, in agreement with studies by Fronticelli et al. (16); at high protein concentrations (favoring Hb tetramers), the functional properties of Hb A and rHb A are similar as described previously (17, 18). The K4,2 values for rHb βF41Y and rHb βF41Y,K66T were 3- and 6-fold decreased, respectively, compared with that for control Hb A (Table I).

Autoxidation—At 37 °C, the oxidation rate of rHb βF41Y,K66T under 1 atm of oxygen was increased by 20% compared with that of native Hb A. Under air, the rHb βF41Y,K66T, which is less oxygen-saturated than Hb A, exhibited a 2-fold increase in oxidation rate relative to Hb A.

Oxygen Equilibrium Curves—Fig. 1 shows the experimental Hill plots obtained for Hb A, rHb βF41Y, and rHb βF41Y,K66T in the absence (Fig. 1A) or in the presence of chloride anions (Fig. 1B). Table II displays the values of the oxygen binding parameters for Hb A, the natural Hb Chico (data of Bonaventura et al. (7)), rHb βF41Y (1), and the double mutant rHb βF41Y,K66T for the liganded form of Hb A, rHb A, rHb βF41Y, and rHb βF41Y,K66T.

The dissociation constants K4,2 were determined by the Hb concentration dependence of peak positions on a Superose-12 HR10/30 column. 10 μl of an Hb solution (2–500 μM on a heme basis) was injected and eluted at a flow rate of 0.4 ml/min. Experiments were performed at 25 °C in Tris acetate buffer, pH 7.5.

| Hb          | K4,2 (μM) |
|-------------|-----------|
| Hb A        | 0.61      |
| rHb A       | 1.41      |
| rHb βF41Y   | 0.54      |
| rHb βF41Y,K66T | 0.26    |

FIG. 1. Oxygen equilibrium curves for Hb A (1), rHb βF41Y (2), and rHb βF41Y,K66T (3) in the absence (A) and in the presence (B) of 100 mM NaCl.
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The partial additivity of the two mutations (decreased for rHb) no longer additive in the presence of effectors. The values of $b_{Hb}$ A and $b_{Hb}$ by about 1.5-fold relative to that of rHb $b_{Hb}$.

**Bohr effects were in the normal range of values (Table III).**

*In the absence of chloride, experiments were performed in 0.01 M Hepes buffer, pH 7.2.*  
*Data from Bonaventura et al. (7) normalized in relation to our values.*

**Table II**

| Species | $P_{50}$ (mm Hg) | $n_{50}$ | $\Delta log P_{50}$ |
|---------|------------------|---------|---------------------|
| Hb A    | 1.8              | 2.3     | 0.52                |
| rHb $\beta F41Y$ | 2.7              | 2.1     | 0.18                |
| rHb $\beta F66T$ | 3.9              | 2.6     | 0.34                |
| rHb $\beta F41Y,K66T$ | 4.7              | 1.4     | 0.42                |

From the experimental curves (Fig. 1), the allostERIC parameters ($L$, $K_T$, and $K_R$) were obtained after fitting the experimental curves to the equation of the two-state allosteric model (19) by using a nonlinear least-squares procedure. $K_T$ and $K_R$ (mm Hg) are the oxygen dissociation constants for the R and T states, respectively; $L$ is the allosteric constant ($T_0/R_0$); $c = K_T/K_R$; the switchover point $i_s$ was calculated as $-\log L/\log c$; % T3 is the amount of triply liganded T state species calculated as $(Lc)^n/(1 + Lc)$. The standard error per point was typically 0.003--0.006.

**Table III**

| Species | $\Delta log (P_{50})$ (1 min, 2,3-DPG) | Bohr effect ($\Delta log (P_{50})$ vs pH) | $\Delta log (P_{50})$ vs [Cl$^-$], pH 7.2 |
|---------|---------------------------------------|----------------------------------------|-----------------------------------------|
| Hb A    | -0.51                                 | -0.51                                  | -0.51                                   |
| rHb $\beta F41Y$ | -0.48                                 | -0.48                                  | -0.48                                   |
| rHb $\beta F41Y,K66T$ | -0.48                                 | -0.48                                  | -0.48                                   |

**Addotic parameters for Hb A, rHb $\beta F41Y$, and rHb $\beta F41Y,K66T$**

In the absence of chloride, the oxygen affinity of the rHb $\beta F41Y,K66T$ was decreased 2.5-fold relative to that of control Hb A and by about 1.5-fold relative to that of rHb $\beta F41Y$. The shift in log $P_{50}$ was 0.34 for Hb Chico, 0.18 for rHb $\beta F41Y$, and 0.42 for the double mutant rHb $\beta F41Y,K66T$, thus showing a partial additivity of the two mutations ($\Delta log P_{50} = 0.52$ for a maximal additivity effect). The Hill coefficient at half-saturation ($n_{50}$), an index of oxygen binding cooperativity, was more decreased for rHb $\beta F41Y,K66T$ than for rHb $\beta F41Y$.

In the presence of chloride, the double mutant rHb $\beta F41Y,K66T$ still exhibited an oxygen affinity lower than Hb A, Hb Chico, and rHb $\beta F41Y$. Nevertheless, the $\Delta log P_{50}$ for rHb $\beta F41Y,K66T$ was equal to 0.45 (0.32 in the presence of 2,3-DPG). This indicates that the effects of the two mutations were no longer additive in the presence of effectors. The values of $\Delta log P_{50}$, corresponding to the oxygen-linked heterotropic allosteric effectors, showed that the chloride, 2,3-DPG and alkaline Bohr effects were in the normal range of values (Table III).

From the experimental curves (Fig. 1), the allostERIC parameters were fitted to the equation of the two-state allosteric model (19) to obtain the oxygen dissociation constants $K_T$ and $K_R$ for the T and R states, respectively (Table IV).

In all experimental conditions, the equilibrium curves could be simulated with values of $K_T$ and $K_R$ for rHb $\beta F41Y,K66T$ and rHb $\beta F41Y$, similar to those for Hb A, indicating that the mechanism of the low oxygen affinity was mainly due to the change in the allosteric equilibrium. The allosteric parameter $L$ was higher for rHb $\beta F41Y,K66T$ than for Hb A and rHb $\beta F41Y$. The switchover point indicates that the allosteric transition T $\rightarrow$ R for rHb $\beta F41Y,K66T$ occurs at a higher oxygen saturation level than for Hb A and rHb $\beta F41Y$. The calculated amount of rHb $\beta F41Y,K66T$ in the T state for tetramers with three ligands was considerably increased: 85% versus 38 and 16% for rHb $\beta F41Y$ and Hb A, respectively. This large increase in the fraction of T state is due to the presence of the mutation $\beta K66T$.

**Kinetic Studies**—Fig. 2 shows the recombination traces of CO after photodissociation. For rHb $\beta F41Y,K66T$ as for Hb A, the traces were biphasic as expected for tetrameric Hb. At low CO photodissociation levels (5%), the CO recombination kinetics of Hb A were fast and monophasic because in these conditions the majority of the photodissociated tetramers are triligated and remain in the R state. In the same conditions, the CO recombination kinetics of rHb $\beta F41Y,K66T$ still exhibited some slow phase. These results suggest, as for the oxygen equilibrium curves, that the allosteric equilibrium of partially liganded species of rHb $\beta F41Y,K66T$ are displaced toward the T state relative to Hb A.

The oxygen association and dissociation rates for Hb A and rHb $\beta F41Y,K66T$ were similar (Table V). These results are
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consistent with the equilibrium data that show that the value of $K_R$ for rHb $\beta F41Y,K66T$ was similar to that of Hb A. However, the CO kinetics suggest a change in the R state properties as well as the shift in allosteric equilibrium.

**DISCUSSION**

In order to obtain a modified Hb that could be used as a Hb-based artificial oxygen carrier, we have used two different strategies to decrease the oxygen affinity of human Hb. We first introduced a mutation in the allosteric interface ($\beta F41Y$) to shift the equilibrium toward the T state by inducing an additional hydrogen bond in the T state conformation, without perturbing the R state conformation. To further decrease the oxygen affinity, we have then investigated the association of this mutation with a second substitution known to have a similar effect, but due to another mode of action.

In normal human Hb, the Phe-$\beta 41$ belongs to a cluster of three highly conserved phenylalanine residues, $\beta 41(C7)$, $\beta 42(CD1)$, and $\beta 45(CD4)$, that participate in the formation of the hydrophobic heme pocket; in Hb A, the Phe-$\beta 41$ has contacts with the heme moiety and is critical to the structural integrity and function of the Hb molecule. Decreased oxygen affinity was observed for the naturally mutated Hb Denver Phe-$\beta 41$ → Ser (20) and Hb Bruxelles deletion of Phe-$\beta 41$ (21). Hb Denver is an unstable Hb variant; the smaller serine residue may impede movement during the allosteric transition of the FG corner of the $\alpha$ subunits along the $\alpha$ helix of the subunits (20). The mutated Hb corresponding to naturally occurring Hb Mequon (2) was synthesized after site-directed mutagenesis to study the functional properties of the pure form (1). The working hypothesis of the $\beta F41Y$ mutation was to induce an additional hydrogen bond in the T state conformation without perturbing the R state conformation. If the $\beta F41Y$ tyrosyl residue forms a new hydrogen bond to the carbonyl of the $\beta 67$ (FG4) histidine residue within the same $\beta$ chain, then the deoxy conformation may be stabilized. While the oxidation rate and heat stability are similar to those of Hb A, this Hb exhibited a 2-fold decrease in oxygen affinity compared with that of Hb A, due to a shift in the allosteric equilibrium.

When two effects have an independent mode of action, the combined effects may be additive. In general, a partial additivity is observed, as shown for the free energy of dimer-tetramer equilibrium for Hb mutants (22). We thus associated the $\beta F41Y$ mutation with a substitution of a residue on the distal side of the heme ($\beta K66T$) to directly act on the heme environment. The abnormal Hb Chico, Lys-$\beta 66$ → Thr, displays a significantly decreased $O_2$ affinity (6, 7). The $\beta 66$ residue is not involved in the subunit interface and is not expected to have a direct effect on the subunit dissociation. X-ray analysis of the deoxy conformation of Hb A shows that Lys-$\beta 66$ makes an ionic bond (salt bridge) between its $\epsilon$ amino group and the carboxyl group of propionate-$7$ of the heme (4). The disruption of the ionic bond provided by Lys was first suspected to be the cause of the altered $O_2$ binding. Other single amino acid substitutions have been investigated to date by site-directed mutagenesis to study the functional properties of the pure form (1). The working hypothesis of the $\beta F41Y$ mutation was to induce an additional hydrogen bond in the T state conformation without perturbing the R state conformation. If the $\beta F41Y$ tyrosyl residue forms a new hydrogen bond to the carbonyl of the $\beta 67$ (FG4) histidine residue within the same $\beta$ chain, then the deoxy conformation may be stabilized. While the oxidation rate and heat stability are similar to those of Hb A, this Hb exhibited a 2-fold decrease in oxygen affinity compared with that of Hb A, due to a shift in the allosteric equilibrium.

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**FIG. 2. Kinetics of CO recombination to Hb A and rHb $\beta F41Y,K66T$.** Relative to either single mutant, the double mutant shows more of the slow phase, characteristic of CO binding to the deoxy conformation. This demonstrates an additive effect of the two mutations. The addition of inositol hexaphosphate (IHP) provides an additive effect of further increasing the slow fraction as well as a decrease in the rate of the slow phase; while less evident from oxygen equilibrium data, the mutations also show an additive effect with the external effectors.

**FIG. 3. Three-dimensional structure of Hb A in the R state showing the sites Phe-$\beta 41$ (in white) and Lys-$\beta 66$ (in blue).** The image was obtained using the VISP program (de Castro and Edelstein, University of Geneva, Switzerland) with a Silicon Graphics 4D/25G workstation. The Hb A crystallographic coordinates were taken from the file 1HHO (Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ) for the oxygenated quaternary structure.

**TABLE V**

| Rate constants | Association | Dissociation |
|----------------|-------------|--------------|
|                | Fast        | Slow         | Fast        | Slow         |
| CO             |             |              |             |              |
| Hb A           | 6.5         | 0.15         | 0.007       | 0.0042       |
| rHb $\beta F41Y,K66T$ | 3.5         | 0.15         | 0.019       | 0.0071       |
| Oxygen         |             |              |             |              |
| Hb A           | 72          | 35           | 25          | 8.9          |
| rHb $\beta F41Y,K66T$ | 72          | 35           | 25          | 6.7          |
values vary greatly for this parameter. This is in part due to the influence on the solvent conditions; e.g. $K_{4,2}$ is about 0.2 $\mu M$ at low ionic force but increases to 1 $\mu M$ at 100 mM NaCl. Protein folding may also play an important role, since the values reported for rHb are often twice that of native Hb A.

For mutant Hbs, the value of the tetramer dissociation constant may be a useful probe of the stability of the protein (11, 22). In addition to an altered oxygen affinity, Hb Chico Lys-β66 → Thr shows an increase in the fraction dimer and in the autoxidation rate (6, 7). However, we observed a decrease in the $K_{4,2}$ value for the association of both mutations F41Y and K66T. The $K_{4,2}$ value for liganded rHb β41Y is decreased 3-fold compared with that of rHb A (Table I), and an additional decrease of a factor of 2 was observed for the double mutant.

Quantification of the additivity requires a choice of parameters. The $P_{50}$ value is one obvious choice for its simplicity and reliability of measurement; however, it depends on several microscopic parameters that determine the intrinsic affinity and allosteric equilibrium. An increase due to the allosteric transition could be compensated by a decrease in $K_T$ or an increase in the fraction dimers. In the absence of external effectors, the rHb β41Y,K66T exhibits a lower oxygen affinity than for either single mutation; the effects of the mutations are therefore probably due to two independent mechanisms. One can also consider the combined effects of the mutations with the effectors such as 2,3-DPG or inositol hexaphosphate; with these effectors, the rHb $P_{50}$ value is one obvious choice for its simplicity and reliability of measurement.

The two residues have no direct contact, as illustrated in Fig. 3, and in principle do not have a correlated participation in their effects on the oxygen affinity. The partial additivity of their effects on the intrinsic oxygen affinity of rHb βF41Y,K66T is therefore probably due to two independent mechanisms. One can also consider the combined effects of the mutations with the effectors such as 2,3-DPG or inositol hexaphosphate; with 2,3-DPG or inositol hexaphosphate, the additivity is less pronounced (Fig. 2). This does not imply that the two effects are no longer additive but rather that the change in $P_{50}$ may no longer have the same magnitude. The flash photolysis kinetics are sometimes more sensitive to changes in the allosteric equilibrium for a late switchover point, e.g. when the transition from T to R occurs only after binding the third ligand. Low photodissociation levels can isolate the reaction for binding the fourth ligand.

The ligand binding properties of this new rHb demonstrate that several mutations may be introduced to obtain combined effects on the oxygen affinity. This would eliminate the need for an external effector, such as 2,3-DPG, as required for an Hb-based blood substitute. However, lower oxygen affinities are often accompanied by an increased autoxidation rate. Unless these parameters can be decoupled, one must accept a compromise of the best oxygen affinity and lowest autoxidation rate (28). The present results show that Hb can be genetically engineered to regulate the oxygen affinity. By associating several smaller changes, the perturbations in the protein stability can be minimized. This appears to be the case for the present study of two mutations in the Hb β chain, one inducing a shift in the allosteric equilibrium ($\beta 41$ mutation) and the other inducing a decrease in the intrinsic oxygen affinity ($\beta 66$ mutation). There is no conflict in accommodating both changes, and an overall additive effect is observed.

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