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Two mutations in recombinant Hb β F41(C7)Y, K82 (EF6)D show additive effects in decreasing oxygen affinity

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Abstract

Based on the properties of two low oxygen affinity mutated hemoglobins (Hb), we have engineered a double mutant Hb (rHb βYD) in which the βF41Y substitution is associated with K82D. Functional studies have shown that the Hb $\alpha_2\beta_2$(C7)F41Y exhibits a decreased oxygen affinity relative to Hb A, without a significantly increased autooxidation rate. The oxygen affinity of the natural mutant βK82D (Hb Providence-Asp) is decreased due to the replacement of two positive charges by two negative ones at the main DPG-binding site. The functional properties of both single mutants are interesting in the view of obtaining an Hb-based blood substitute, which requires: (1) cooperative oxygen binding with an overall affinity near 30 mm Hg at half saturation, at 37 °C, and in the absence of 2,3 diphosphoglycerate (DPG), and (2) a slow rate of autooxidation in order to limit metHb formation. It was expected that the two mutations were at a sufficient distance (20 Å) that their respective effects could combine to form low oxygen affinity tetramers. The double mutant does display additive effects resulting in a fourfold decrease in oxygen affinity; it can insure, in the absence of DPG, an oxygen delivery to the tissues similar to that of a red cell suspension in vivo at 37 °C. Nevertheless, the rate of autooxidation, 3.5-fold larger than that of Hb A, remains a problem.

Keywords: allostery; blood substitute; hemoglobin; mutagenesis

The segments of the hemoglobin polypeptide chains that are mainly involved in the stabilization of the T quaternary structure leading to a low oxygen affinity of the tetrameric form are located at: (1) the allosteric $\alpha_1\beta_2$ interface (the CD and EF corners); (2) the N- and C-termini of the subunits; and (3) the binding sites of anions in the central cavity of the protein (Bonaventura et al., 1976; Dickerson & Geis, 1983; Perutz, 1970). The heme pocket also plays a significant role in determining the heme geometry changes when oxygen binds to the iron.

For many years, biochemical studies have been carried out to elaborate an Hb-based blood substitute (Bunn, 1993). More recently, the use of site-directed mutagenesis has permitted production of recombinant human Hb whose oxygen-binding properties and heterotropic effects can be varied (Nagai et al., 1985; Pagnier et al., 1986). The main physiological requirements for an adequate transport of oxygen by a solution of Hb are the cooperativity in ligand binding, and an oxygen affinity two- to threefold lower than that of stripped human Hb. The latter is due to the absence, in the plasma, of 2,3-diphosphoglycerate, the natural effector of human Hb. Within the red blood cells, DPG contributes to lower the oxygen affinity by a factor of two, at 37 °C, in physiological conditions of pH and ionic strength. Naturally occurring mutations may be reproduced at various sites in the molecule to mimick the effect of DPG.

We have previously shown that the substitution in Hb A of a tyrosyl residue for a phenylalanine at position β(C7)41 leads to a low oxygen affinity hemoglobin, with a well-preserved cooperativity in oxygen binding and without increasing the rate of autooxidation. The mechanism of these peculiar properties have been attributed to an additional hydrogen bond induced by the F → Y substitution at the $\alpha_1\beta_2$ interface. The increase in P_{50} for F41Y relative to Hb A could be attributed to a change in the allosteric constant, L_0, whereas the oxygen equilibrium dissociation constants for the T and R states, K_T and K_R respectively, were not modified (Baudin et al., 1992).

In the interest of further decreasing the oxygen affinity of this rHb, we chose to add βK82D, the mutation of Hb Providence-

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Abbreviations: Hb, hemoglobin; rHb, recombinant human Hb; OEC, oxygen equilibrium curve; DPG, 2,3-diphosphoglycerate; RSR4, 2-[4-[[(3,5-dichloroanilino)carbonyl]-methyl]phenoxy]-2-methylpropionic acid.

Asp (Bonaventura et al., 1976), as a second mutation in the β chains. In Hb Providence-Asp, the mutation results in the replacement of two positive charges by two negative ones at the main DPG site, in the central cavity. The effect may be compared to the neutralization of the positive charges by DPG or chloride anions, which stabilizes the *T* structure, and consequently lowers the oxygen affinity. We expected that the two mutations were at a sufficient distance, about 20 Å according to the 3D representation of the tetramer, that their effects would be additive (LiCata & Ackers, 1995; Wajcman et al., 1995). The double mutant displays a fourfold decrease in oxygen affinity, and a rate of autooxidation 2- to 3.5-fold larger than that of Hb A. The mechanism of these effects may be discussed from the analyses of the allosteric properties of the double mutant.

Results

Analysis of the purified rHb βYD by isoelectric focusing showed that it migrated as a single band, in a position more anodical relative to Hb A (pI = 6.8 and 6.98, respectively). This difference in pI is low, considering the mutation of an Asp for a Lys. As expected, it is identical to that observed for purified Hb Providence-Asp (pI = 6.8) compared to Hb A, because the pI for the rHb βF41Y and Hb A are identical (Baudin et al., 1992).

The fluorescence emission of rHb βYD at 330 nm (maximum intensity of fluorescence of buried tryptophan within a protein) is similar to that for Hb A. This result confirms a correct refolding for the recombinant Hb with no heme loss. The UV/visible spectra characteristics of carboxylated and oxygenated forms for rHb βYD are also identical to Hb A. In particular, the ratio of absorbance intensity between the Soret band and the UV peak at 280 nm is equal to 5, the normal value for HbCO, and is in agreement with the fluorescence results, which show no heme loss from rHb.

Oxygen equilibrium analyses

Table 1 gives the values of the oxygen equilibrium data for Hb A, rHb βF41Y, natural mutant Hb βK82D, and the double mutant rHb βYD in the presence and in the absence of chloride.

In the absence of chloride, an experimental condition that allows a determination of the intrinsic functional properties of the mutants, all three modified hemoglobins exhibit a decreased oxygen affinity relative to Hb A. The shift in P_{50}, expressed as $\Delta \log P_{50}$ relative to Hb A, is 0.17 for rHb βF41Y, 0.44 for Hb βK82D, and 0.75 for the double mutant rHb βYD, thus slightly more than predicted from a simple additivity ($\Delta\log P_{50} = 0.61$). In these experimental conditions, the n_{50} values, an index of cooperativity in oxygen binding, are similar to that for control Hb A.

In the presence of chloride, the three mutants still exhibit P_{50} values higher than Hb A. The additivity is nearly perfect in this case, with $\Delta \log P_{50}$ values (at pH 7.2) of 0.28 for rHb βF41Y, 0.23 for Hb βK82D, and 0.54 for the double mutant. In these conditions, the mutation βF41Y makes the larger contribution to the high P_{50} of the double mutant. The low effect of chloride anions on the oxygen affinity of Hb βK82D results in a smaller $\Delta\log P_{50}$ relative to Hb A for solutions with chloride anions.

Figure 1 illustrates the dependence of $\log P_{50}$ as a function of log chloride concentration. Compared to normal Hb A, Hb Providence-Asp exhibits a high P_{50} in the absence of chloride. The difference in oxygen affinity for these two Hbs decreases at elevated chloride concentration, with a convergence estimated by extrapolation near 1 M Cl^-. This results in a large decrease in the chloride oxygen-linked effect by a factor of 2 for Hb Providence-Asp (Table 2). The chloride titration curve for the βYD mutant is parallel to that of Hb Providence-Asp with higher P_{50} values.

Compared to Hb A, the n_{50} values for the three mutant Hbs are decreased. The heterotropic effects for each of these hemoglobins are given in Table 2. As expected rHb βYD displays a low oxygen-linked chloride effect and a low alkaline Bohr effect, as observed for Hb Providence-Asp.

The heterotropic effects of RSR-4, a strong effector that binds to the α subunits (Abraham et al., 1992), on rHb βYD and Hb A are similar (Table 2). Because the lysyl residue at position 82 in the β chains plays an important role for the binding of DPG (Arnone, 1972), its replacement by an aspartyl residue induces a drastic decrease in the binding constant for this effector. This is in agreement with the loss of the organophosphate effects in

Table 1. *Oxygen binding parameters for Hb A, rHb β F41Y, Hb Providence-Asp (β K82D), and rHb β YD*[a]

	Hb A		Hb βK82D		rHb βF41Y		rHb βYD		$\Delta\log P_{50}$[b]		
pH	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}	βK82D	βF41Y	βYD
$Cl^- = 0$[c]											
7.2	1.8	2.0	5.0	2.1	2.7	2.1	10.3	2.2	0.44	0.17	0.75
Cl^- = 0.1 M											
6.5	12.2	2.5	14.1	1.9	22.5	1.8	28.7	1.7	0.06	0.26	0.37
7.2	5.1	2.8	8.7	2.3	9.7	2.1	17.2	2.0	0.23	0.28	0.54
7.8	2.3	2.1	5.7	2.1	4.0	2.1	11.0	2.1	0.44	0.17	0.75

[a] Other conditions: bis-Tris or Tris 0.05 M, catalase 20 μg/mL, EDTA 50 μM, [heme] 70 μM, 25 °C. The percentages of metHb formation at the end of the oxygen equilibrium curve recordings were found between 2 and 5%, except for βYD at pH 7.8, where it was 10%.

[b] Shift in P_{50}, expressed as $\Delta \log P_{50}$ relative to Hb A.

[c] Hepes 0.01 M.

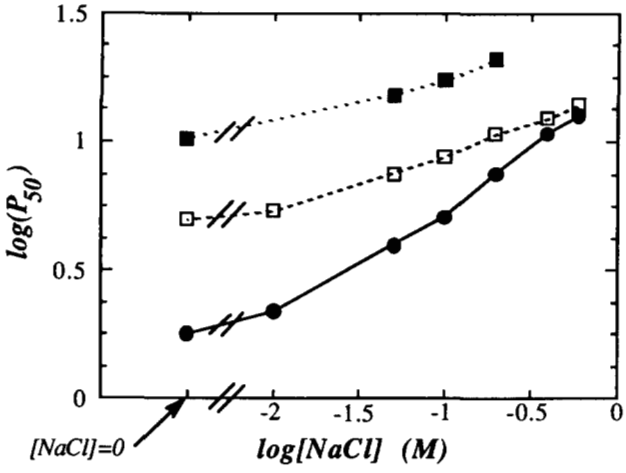

Fig. 1. Variations of the oxygen affinity (log P_{50}) of rHb βYD (■), natural Hb Providence-Asp, βK82D (□), and Hb A (●) with increasing concentration of chloride. P_{50} values were obtained from oxygen equilibrium curves as described in the Materials and methods. Other conditions were pH 7.2, bis-Tris buffer 50 mM (except for conditions without chloride, where 10 mM Hepes buffer was used), catalase 20 μg/mL, EDTA 50 μM, 25 °C.

native Hb Providence-Asp (Bonaventura et al., 1976; Bardakdjian et al., 1985).

Analysis of the equilibrium curves

Figure 2 (A: without chloride, B: with chloride) illustrates the experimental Hill plots obtained for the different Hbs studied. From these data, we calculated the allosteric parameters for each mutated rHb compared to native HbA. These were performed after fitting the experimental points to the equation of the two-state allosteric model (Monod et al., 1965). In cases where the allosteric equilibrium is greatly shifted toward the T-state ($i_s >$ 2.8), there is some cross-relation between the parameters K_R and L. The binding of the fourth ligand is no longer a pure R state ($R3$ to $R4$) process, and the apparent shift in the upper asymptote can be simulated by an increase in K_R or L; the oxygen-binding curves can be simulated by various simulations of these two parameters (Marden et al., 1990). In these cases, there is some influence from the kinetic data to better determine K_R. The results are given in Table 3.

In the absence of chloride, the low oxygen affinity of the βYD mutant is due in major part to the high value of the allosteric constant L, but also to a twofold increase of the intrinsic equilibrium constants K_R and K_T (Table 3). Compared to Hb A, these differences in oxygen binding involve the specific effects of the two single mutations: whereas βF41Y shows mainly a change in the allosteric parameter L (no change in K_R and K_T), βK82D displays changes both in the allosteric equilibrium constant and in the intrinsic oxygen affinities. As discussed above, the shift in P_{50}, expressed as $\Delta \log P_{50}$ (see Table 1) indicates that both effects occur in the double mutant.

In the presence of chloride, and due to the small effect of these anions to the Hb variants that carry the mutation K82D (Table 3), the oxygen affinity of βYD mutant is mostly accounted for by the large increase in L and, to a lower extent, the change of K_T.

Kinetic studies

Measurements of CO bimolecular kinetics after flash photolysis are a sensitive tool to estimate the allosteric equilibrium of an Hb sample at various fractions of photodissociated heme-CO. The partial allosteric transition $R \rightarrow T$ after flash photodissociation of CO for Hb A and rHb βYD implies that the bimolecular kinetics of CO rebinding are biphasic. The R state tetramers rebind CO 30-fold faster than T-state tetramers. Figure 3 shows that the allosteric $R \rightarrow T$ transition is twofold more pronounced for rHb βYD (60% slow phase) than for Hb A (35%). At low CO photodissociation levels, the difference in the fraction slow phase between mutant and Hb A, increases. For levels below 10%, the bimolecular kinetics of Hb A are nearly monophasic and fast because the majority of the photodissociated tetramers are triliganded, which remain in the R-state. In identical conditions, the CO kinetics for rHb βYD exhibit 15% of the slow phase (Fig. 3). These results clearly show that the allosteric equilibrium of partially liganded species for rHb βYD are displaced toward the T quaternary structure relative to Hb A. The intrinsic rate coefficients of CO binding to Hb A and to the mutants in the R state are similar ($k_{on} = 6 \times 10^6$/M/s; $k_{off} = 0.012$/s). However, the stopped-flow results indicate that the oxygen dissociation rate from the fully liganded form was twofold higher, with a rate of 50/s for the mutants with the K82D substitution. Based on these kinetic results, we reanalyzed the equilibrium curves using the higher value for K_R for Hbs βK82D and βYD. The T-state oxygen affinities (K_T) are based only on the equilibrium data and, in the absence of chloride ions, show even larger changes than those of K_R (Table 3). The relative stabilization of the T-state conformation for the mutants, as indicated by the CO bimolecular kinetics, is confirmed by the increase in L, consistent with a higher switch-over point (i_s). The consequence is a higher fraction of $T3$ species for the mutants relative to Hb A (Table 3).

Table 2. *Heterotropic effects for Hb A, rHb β F41Y, Hb Providence-Asp (β K82D), and rHb β YD*

Effectors	HbA	Hb βK82D	rHb βF41Y	rHb β YD
Chloride (pH 7.2) $\Delta \log P_{50}/\Delta \log[Cl^-]$	0.48	0.23	0.50	0.23
Bohr effect (Cl^- = 0.1 M) $\Delta \log P_{50}/\Delta$pH	−0.54	−0.31	−0.53	−0.32
RSR4 effect (Cl^- = 0) $\Delta \log P_{50}/\pm$RSR4 0.5 mM	0.92	nd	nd	0.86

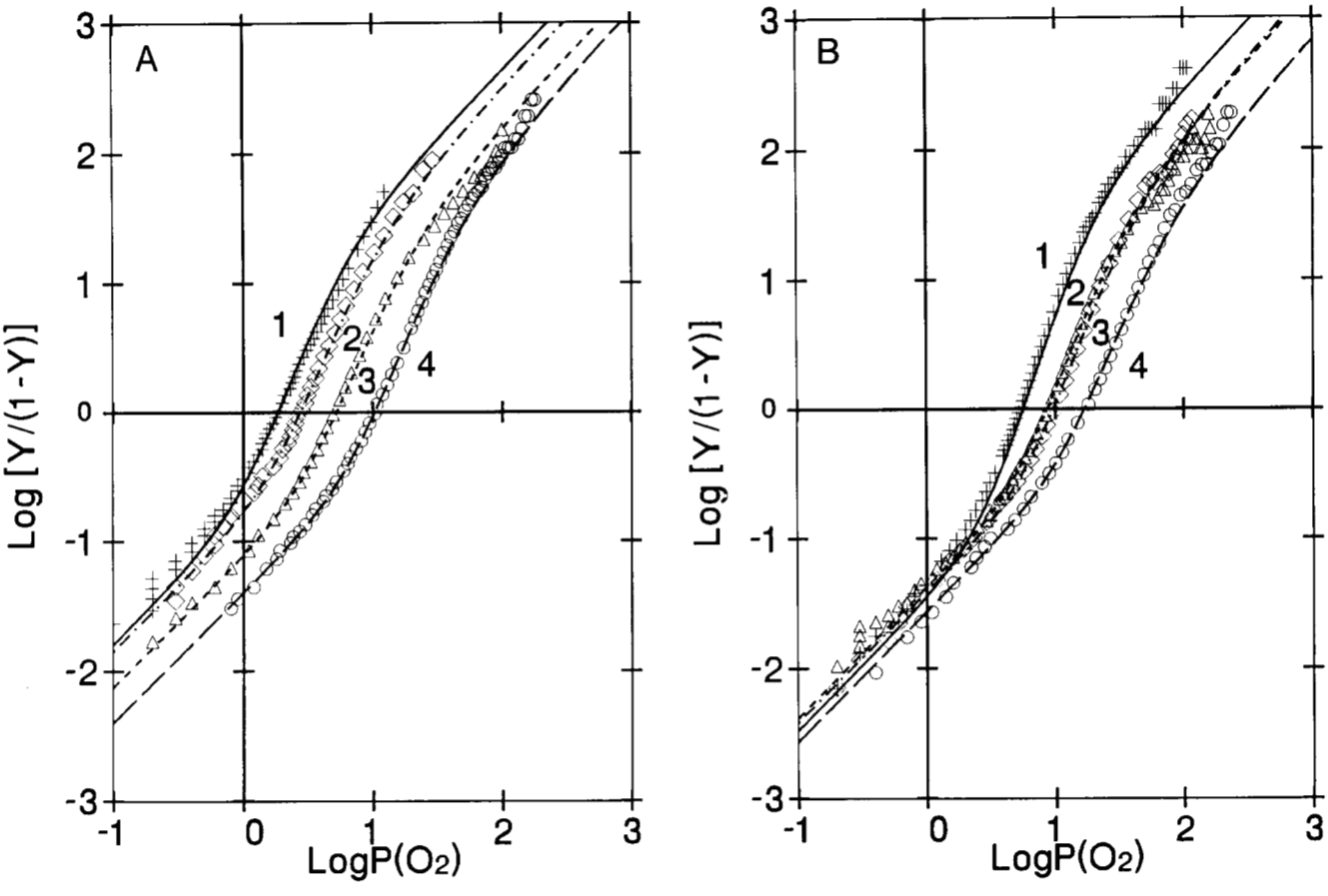

Fig. 2. Oxygen equilibrium curves (Hill plots) for Hb A (1), rHb βF41Y (2), Hb Providence-Asp βK82D (3), and rHb βYD (4). **A:** in the absence of chloride; **B:** in the presence of 100 mM NaCl. Symbols represent one of six experimental data points. The lines are obtained after nonlinear least-squares fitting to the two-state model (Monod et al., 1965) using the parameter values given in Table 3. Other experimental conditions are as indicated for Figure 1.

Autooxidation

At 37 °C, the oxidation rate of rHb βYD under 1 atm O_2 (k_{ox} = 0.045 h^{-1}) is a factor of two higher than that of Hb A; under air, the mutated rHb is not saturated with the ligand, and the autooxidation rate is 3.5-fold increased (Fig. 4). Oxidation studies of myoglobin mutants revealed that there is an inverse correlation between the oxidation rate and the oxygen affinity at

Table 3. *Allosteric parameters for Hb A, rHb β F41Y, Hb Providence-Asp (β K82D), and rHb β YD*

Hb	[Cl^-] (mM)	L	c	K_R (mm Hg)	K_T (mm Hg)	%T_3[a]	i_s[b]	σ[c] (× 10^3)
Hb A	0[d]	7.0×10^3	0.030	0.19	6.3	16	2.5	4.5
rHb βF41Y	0	3.0×10^4	0.027	0.19	7.0	37	2.8	4.4
Hb βK82D	0	3.0×10^4	0.027	0.36	13.3	37	2.8	4.6
rHb β YD	0	4.3×10^5	0.014	0.36	25.0	56	3.0	4.8
Hb A	100	1.0×10^5	0.0097	0.24	24.7	8	2.5	4.3
rHb βF41Y	100	1.2×10^6	0.010	0.26	26.0	54	3.0	5.2
Hb βK82D	100	2.2×10^5	0.015	0.36	24.0	42	2.9	4.5
rHb β YD	100	2.4×10^6	0.010	0.38	36.5	73	3.2	6.1

[a] $\%T_3 = (Lc^3)/(1 + Lc^3)$.
[b] $i_s = -\log L/\log c$.
[c] σ = standard error per point.
[d] Hepes 0.01 M.

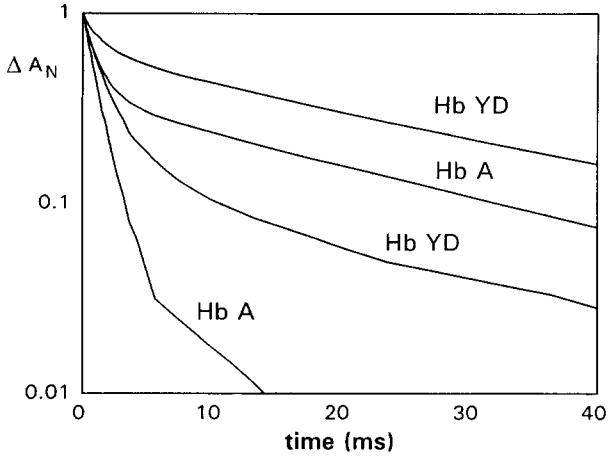

Fig. 3. Normalized kinetics of CO bimolecular recombination at 25 °C. Experimental conditions were: pH 7.2, 100 mM NaCl, 50 mM bis-Tris, 0.1 mM Na dithionite, 0.1 atm CO; heme concentration was 60 μM. Two levels of photodissociation are shown: 7% dissociation for the lower curves; 60% for the upper curves.

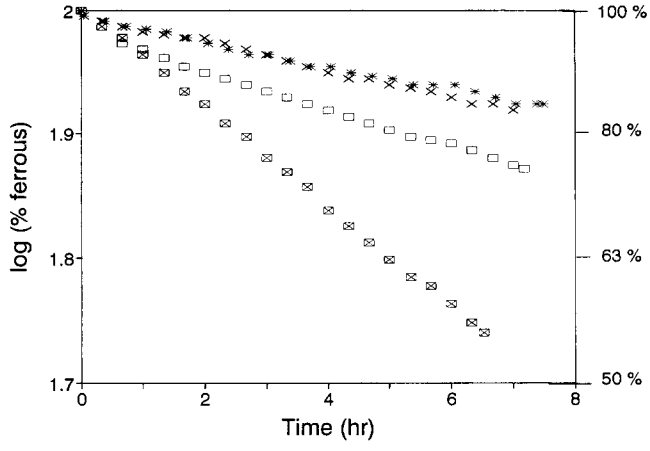

Fig. 4. Autooxidation of oxyhemoglobin solutions equilibrated under air or 1 atm of oxygen at 37 °C in 20 mM phosphate at pH 7.0. Heme concentration was 40 μM. Absorbance spectra were recorded every 20 min during 7 h. ⊠, rHb βYD under air; □, rHb βYD under 1 atm O2; ×, Hb A under air; ✳, Hb A under 1 atm O2.

equilibrium (Brantley et al., 1993). In spite of the complexity of the autooxidation mechanism of the heme, it is clear that the lower the oxygen affinity of Hb, the faster the autooxidation rate. The oxidation rate of rHb βF41Y is similar to that of Hb A, whereas its oxygen affinity is twofold lower (Baudin et al., 1992). A plausible explanation for these results is that there is no change of the K_R value for rHb βF41Y, whereas the additional mutation at position β82 leads to an increase of the K_R value. As a result, the increase of oxidation rate for rHb βYD under 1 atm oxygen is due to the higher K_R, and the further increase for samples under air is due to the fact that the Hb is not saturated.

Discussion

What are the appropriate modifications altering the functional properties of human Hb in order to elaborate a physiological oxygen carrier? Mutations changing only the allosteric equilibrium at each step of oxygen binding toward the T-state seem to be the most useful. It is not advantageous to decrease the oxygen affinity beyond a certain limit, because the Hb would not be saturated with ligand; this would lead to less efficient oxygen delivery and increased autooxidation rate.

It is possible to adjust by mutagenesis the oxygen-binding properties of Hb using low oxygen affinity variants as models. If two mutations are not in direct contact, their functional effects tend to be additive (LiCata & Ackers, 1995, Wajcman et al., 1995). The additive effects of several mutations on the binding sites of an oligomeric protein such as hemoglobin are not always predictable. The P_{50} value is a complex function of the allosteric parameters; however, because it is well determined, it can serve as a sensitive test of the additivity of the individual effects.

Oxidation of the hemes is a general problem for low oxygen affinity mutants: the rate is correlated with the oxygen affinity, but remains low compared to cases when the heme is not protected by the globin. The oxidation problems therefore tend to be worse for substitutions in the heme pocket, because of the risk of inducing heme loss or promoting the entry of oxidative catalysts into the heme site (Brantley et al., 1993). Rather than decreasing the intrinsic affinity of the *R*- and *T*-states, which would increase the rate of autooxidation, it seems preferable to maintain the same K_R and to decrease the overall oxygen affinity by shifting the allosteric equilibrium toward the *T*-state.

For the double mutant rHb βYD, the P_{50} values show the additivity of the effects of the individual mutations. With a fourfold decrease in oxygen affinity, the rHb βYD could be of interest for the development of an Hb-based blood substitute; but as observed for low-affinity variants, the rate of autooxidation is increased relative to Hb A. The best strategy to obtain a viable compromise for these parameters could be the stabilization of the T quaternary structure by the introduction of several mutations in the critical interface region $\alpha_1\beta_2$.

Materials and methods

The βYD mutation was introduced into the β-globin cDNA by site-directed mutagenesis using a primer synthesized by the phosphotriester method (Genset, Paris, France). The mutated β-globin subunit was produced as a fusion protein in *Escherichia coli* using the expression vector pAT PrcII FX β (Bihoreau et al., 1992). After extraction and purification, the fusion protein was cleaved by digestion with the bovine coagulation factor Xa. The β subunits were folded in the presence of cyanohemin and the partner α subunits to form the tetramer $\alpha_2\beta_2$ (Nagai & Thogersen, 1984). Refolding of recombinant wild-type β subunits synthesized by this method results in Hb tetramers with oxygen-binding properties essentially identical to those of natural Hb A (Nagai et al., 1985; Fronticelli et al., 1991). The ferrous β-subunits were regenerated by reduction using sodium dithionite under 1 atm CO. Purification of the carbon monoxide mutant form was performed as described previously (Nagai

& Thogersen, 1984; Bihoreau et al., 1992). Isoelectric focusing of the carbon monoxide derivatives was performed along a pH gradient from 6.5 to 8.5 with the LKB system. The structure of the β-mutated chains was checked by reverse-phase HPLC of the tryptic digest and amino acid analysis of the abnormal peptide. The concentration of Hb samples was determined by spectrophotometry, taking an ϵ value of 14.3 mM^{-1} cm^{-1} at 540 nm for the CO form of Hb. Static absorption spectra of the Hbs were recorded with a SLM-Aminco DW2000 spectrophotometer. Fluorescence spectra of the Hbs were recorded with 10 μM solutions (on a heme basis) in 10 mM sodium phosphate buffer at pH 7.0 with a SLM-Aminco 8000 spectrofluorometer. With excitation at 280 nm, the emission spectra were recorded for both the buffer and the Hb samples. The fluorescence spectra are a sensitive test of the quality of the refolding of reconstituted hemoproteins, because the tryptophan residues are normally highly quenched (factor of 50) by the heme. Oxygenated Hb samples were analyzed shortly after decarboxylation of the CO forms.

Oxygen equilibrium curves were recorded with a continuous method using a Hemox Analyzer (TCS, Huntington Valley, Pennsylvania). Fifty micromolar EDTA and 20 μg/mL catalase were added to limit oxidation during the recordings. MetHb formation was checked by spectrophotometry after each OEC recording.

The studies on Hb Providence-Asp were performed on the natural Hb βK82D isolated from the hemolysate of a heterozygous propositus by chromatography on BioRex 70 (Biorad) in 1985, further checked by analytical isoelectric focusing, and kept in liquid nitrogen in the CO form. The CO-sample was decarboxylated in ice water under 1 atm oxygen and treated by routine methods. The P_{50} measured in 1995 (9 mm Hg) for that mutant was similar to that in 1985 (11 mm Hg) (Bardakdjian et al., 1985).

The oxygen equilibrium curves were analyzed as described in Kister et al. (1987). The P_{50} and n_{50} values were calculated by linear regression from the Hill equation for oxygen saturation between 40% and 60%. Heterotropic effects were calculated as $\Delta \log P_{50}/\Delta \log$ [effector]. The oxygen equilibrium data were also analyzed in terms of the two-state allosteric model of Monod et al. (1965). The allosteric parameters were obtained by nonlinear least-squares fitting of the experimental OEC's.

The rate of oxidation for liganded Hb samples was measured by spectrophotometry at 37 °C under 1 atm oxygen and under air. Hb solutions were 40 μM in heme, in 20 mM potassium phosphate buffer, pH 7.0. The final metHb spectrum was generated by oxidation with a slight excess of potassium ferricyanide. The analyses of the spectra were made by computer simulations on a large-wavelength domain between 350 and 700 nm.

The bimolecular recombination rates (k^{on}) were measured after flash photolysis with a 10-ns YAG laser pulse delivering 160 mJ at 532 nm (Quantel, France). Samples were in 1-mm optical cuvettes with observation at 436 nm (Marden et al., 1988). Measurements were made at 25 °C, pH 7.2, 0.1 M NaCl, for samples equilibrated under 1 or 0.1 atm for CO and 1 atm for O_2. Traces shown are the average of at least five measurements.

Kinetics of O_2 dissociation by CO replacement were measured with a stopped-flow apparatus at 410 nm (Biologic, France). Because under air the mutated tetramers may not be totally saturated with O_2 before mixing, the oxygenated samples were equilibrated under 1 atm O_2 at least for 20 min before the measurements. Removal of oxygen, which is necessary to prevent the competition between ligand rebinding after dissociation and CO replacement, was made by adding a large amount of sodium dithionite. Experimental conditions were 100 mM NaCl, 50 mM bis-Tris, 15 mM sodium dithionite, pH 7.2, with 50 μM CO and 0.7 mM O_2 at 25 °C. Heme concentration was 10 μM. Dead time before the recording of k_{O2}^{off} kinetics was 5 ms.

The k_{CO}^{off} kinetics were performed with the same apparatus at 540 nm by oxidation of carboxylated Hb samples with potassium ferricyanide. Experimental conditions were 100 mM NaCl, 50 mM bis-Tris, 15 mM potassium ferricyanide, pH 7.2, 25 °C. Heme concentration was 10 μM. The method based on replacement of CO by NO gave similar results compared to oxidation by potassium ferricyanide.

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