Loss of allosteric behaviour in recombinant hemoglobin $\alpha_2\beta_2$92(F8) His→Ala: restoration upon addition of strong effectors

A. Dumoulin[a], L. Kiger[a], R. Jiang[a], V. Baudin[a], C Vasseur[a], S.G. Sligar[b], M.C. Marden[a], J. Pagnier[a,*], C. Poyart[a]

[a]*Institut National de la Santé et de la Recherche Médicale U 299, Hôpital de Bicêtre, 94275 Le Kremlin-Bicêtre, France*
[b]*Beckman Institute, 405 N. Mathews Avenue, Urbana, IL 61801, USA*

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Abstract In the stereochemical model proposed by Perutz [1], the Fe-His(F8) bond plays a significant role in the allosteric transition in hemoglobin and the resulting cooperativity in ligand binding. When this bond is ruptured, there is a loss in the transmission of the information concerning ligand binding; examples are Hb(NO)$_4$ in the presence of inositol hexakisphosphate (IHP), or nickel substituted Hb hybrids which, despite being liganded, exhibit deoxy-like properties. To study the effects of the loss of the iron proximal histidine bond, we have engineered the $\alpha_2\beta_2$(F8)H92A recombinant Hb. The replacement of the highly conserved proximal histidine F8 residue by an alanine results in a low affinity for the heme group and a loss of the allosteric properties; kinetics of CO recombination after photodissociation show only the rapid bimolecular phase, characteristic of the high affinity R-state. However, a significant amount of deoxy (T-state) kinetics are observed after addition of external effectors such as IHP. The iron-histidine bond is apparently crucial for the heme-heme interaction, but the allosteric equilibrium may still be influenced by external constraints.

Key words: Recombinant hemoglobin; Homotropic effect; Heterotropic effect; Proximal histidine; Heme-heme interaction

1. Introduction

The stereochemical model proposed by Perutz [1] provides an elegant explanation for the hemoglobin (Hb) allosteric mechanism. The binding of a ligand to the iron (with modification of its spin state) is the trigger of the Hb allosteric transition. Kinetic studies have shown that after photodissociation of the oxy form, the iron moves within picoseconds [2] towards the heme plane by at least 0.2 Å compared to the deoxy Hb configuration while the Fe-His (F8) bond is shortened by 0.2 Å [3]. The resulting translation of the F helix is transmitted to the $\alpha_1\beta_2$ allosteric interface leading to the modification of electrostatic contacts between the α C helix and the β FG corner [4]. The Fe-His(F8) bond plays the role of an energetic conductor: when a ligand molecule binds to the heme, the chemical energy is distributed to the whole protein. As stated by Perutz [5] ' ... in the T structure nearly all the free energy is stored in the protein rather than in the heme, because the protein is softer and could take up strain energy in hydrogen bonds, Van der Waals interactions, and a variety of small torsions.'

The rupture of the Fe-His(F8) bond has been observed for α subunits of Hb(NO)$_4$ in the presence of inositol hexaphosphate (IHP) [6,7], in (αNiβFe)$_2$ metal substituted hemoglobin [8] or in (αNiβNi)/(αFeβFe) hybrid hemoglobins [9]. The breaking of the bond between the iron and the proximal histidine has also been observed in various carboxyl or nitrosylmyoglobins at low pH [10]. In hemoglobin the rupture induces a shift of the allosteric equilibrium towards the T structure. Interestingly the (αFeβPP)$_2$ hybrid hemoglobin, where proto-porphyrin without the metal atom is substituted for heme, binds oxygen cooperatively, contrary to the (αPPβFe)$_2$ hybrid [11]. This last result raises the question concerning the pathways used by Hb to transmit the information of ligand binding to the other subunits. To study the role of the Fe-His(F8) bond in the cooperative oxygen binding, we prepared the artificial mutant $\alpha_2\beta_2$92(F8)His→Ala (rHb βH92A). It was of interest to know whether cooperative oxygen binding still occurred despite the replacement of the proximal histidine residue by a small, non-polar alanine residue.

Natural hemoglobin mutants at position F8 are unstable and exhibit functional characteristics of semi-hemoglobins, except for hemoglobins M: Hb Hyde Park β(F8)H92Y and Hb Iwate α(F8)H87Y [12]. In these two hemoglobin variants the ferric iron is coordinated with the tyrosinate group [13]. This observation suggests that there is a flexible distance between the porphyrin plane and the F helix which can accommodate an additional hydroxyl group. In deoxy Hb the Fe-Cα F8 distance is equal to 6.5 Å. Similarly in myoglobin (F8)H93G, the loss of the proximal histidine leads to a hole which can be occupied by various hindered bases such as imidazole [14].

We have recorded the static UV/visible absorbance and fluorescence spectra of the carbon monoxide and deoxy forms of recombinant Hb β(F8)H92A to estimate the fraction of dehemeinized β subunits. We have also measured CO recombination kinetics for this mutant to understand the influence of the loss of Fe-His(F8) bond in the β subunits.

2. Material and methods

We produced the mutated β-globin subunit using the pATPrTet [15] expression vector harboring a β synthetic coding sequence with optimal codon usage in *Escherichia coli* [16]. The mutational process is facilitated by the different unique restriction sites placed along this coding sequence and consists of replacing the fragment cleaved by the corresponding synthetic oligonucleotides bearing the mutation. The pATPrTet expression vector directs the synthesis of the fusion protein CIIFX-β globin under the control of the λ Pr promoter. The cloned DNA was checked by complete sequencing. The use of a fusion CII

*Corresponding author. Fax: (1) 46 70 64 46.

Abbreviations: Hb, hemoglobin; IHP, inositol hexakisphosphate; L345, 2-[4-(3,4,5-dichlorophenylureido)phenoxy]-2-methylpropionic acid; PP, protoporphyrin.

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protein is justified by the need of NH_2 terminal methionine removal [17]. The β subunits were folded in the presence of cyanhemin and the partner carbonyl α subunits, prepared from natural HbA, to form the tetrameric Hb $\alpha_2\beta_2$ [15]. The structure of the mutated chain was checked, after amino-ethylation, by high performance liquid chromatography (HPLC) of the tryptic digests and amino acid analysis of the mutated peptide. Cellulose acetate electrophoresis of the mutant Hb at pH 8 was performed according to routine methods. All spectrophotometric studies were performed with a SLM-Aminco DW 2000. Fluorescence studies were recorded using an SLM 8000 spectrofluorometer. Kinetics of CO recombination were recorded after flash photolysis by a 10 ns pulse providing 160 mJ at 532 nm (Quantel-Yag laser) [18]. Hemoglobin concentrations were determined from the absorbance at 540 nm using an extinction coefficient for HbCO of 14.3 mM^{-1} cm^{-1} [19]. We used this wavelength because the extinction coefficient is similar for the carboxy form of α and mutated β subunits and Hb A. For Hb the globin and the heme contribute roughly equally to the absorbance at 280 nm. The fraction of deheminized mutated globin chains in rHb βH92A was estimated using an extinction coefficient of 16 mM^{-1} cm^{-1} at 280 nm for the β-globin.

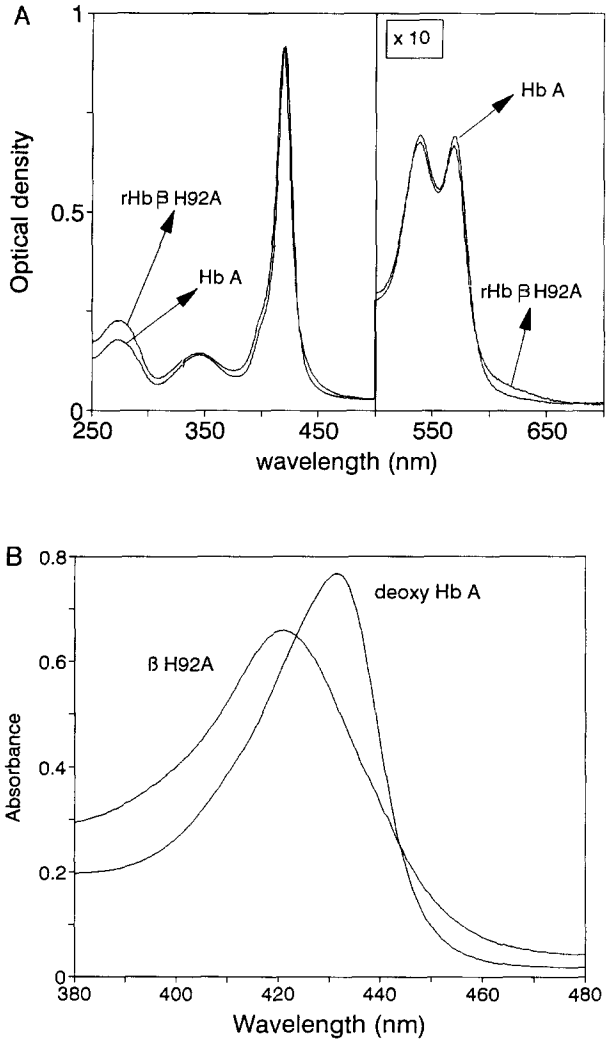

Fig. 1. (A) Absorption spectrum of the carbonyl form of Hb A compared to that of rHb βH92A. The concentration of the HbCO samples was 5 μM (on a heme basis) in 100 mM phosphate buffer pH 7.0. (B) Absorption spectrum of deoxy Hb A and deoxy rHb βH92A, in 100 mM phosphate buffer at pH7.0, 25°C, with 100 μM Na dithionite. Subtraction of a 55% contribution of the Hb A spectrum, to estimate the spectrum for the mutant β chain, revealed a large blue shift in the Soret band for the mutant subunit.

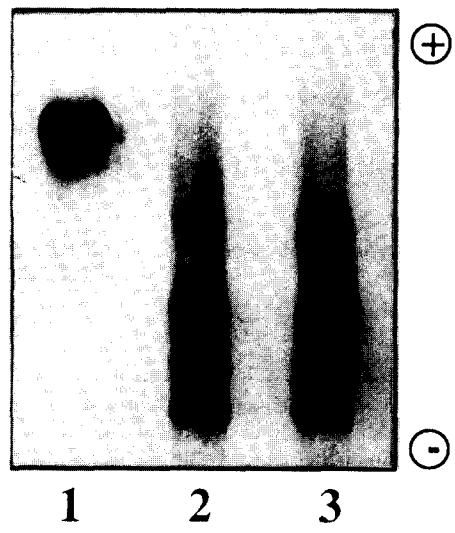

Fig. 2. Electrophoresis on cellulose acetate at alkaline pH. Two bands are present for rHb-CO βH92A (lane 3) migrating between HbCO A (lane 1) and free α-CO chains. The addition of hemin-CN to the mutant Hb (lane 2) removes to a large extent the proportion of the more negative band.

3. Results and discussion

The CO spectrum of rHb βH92A reveals a fraction of deheminized globin. For the same Soret absorption, the band at 280 nm is increased for the mutant compared to normal Hb A (Fig. 1A). The absorption ratio $\varepsilon_{420}/\varepsilon_{280}$ is lowered, from 5.2 for the native HbCO to 4.1 for the mutant, indicating a significant amount of apo-globin. This could also be due to a lower absorption coefficient for the β chain heme as a result of the perturbation in the heme pocket. This means that a large amount of mutant tetramers (about 40%) could be affected by at least one non-functional β chain. Fluorescence results (data not shown) also suggest a loss of heme. The fluorescence spectra are a sensitive probe for the refolding of recombinant hemoglobins, since the fluorescent tryptophan residue is normally highly quenched by the heme. The fluorescence intensity for the mutant is 10-fold stronger than that observed for Hb A, indicating heme loss. It decreases 2-fold upon addition of an excess of hemin suggesting hemin binding to at least half of the apo β-globins.

The deoxy spectra of Hb A and rHb βH92A are shown in Fig. 1B. After normalization at 560 nm, the mutant's Soret band reveals a shift towards the blue and a larger bandwidth. This suggested the presence of two overlapping bands for the mutant. Assuming a normal spectrum for the α chains, we subtracted their contribution using the deoxy spectrum of Hb A. Note that since there is some heme loss from the β chains, the observed spectrum should contain more than 50% contribution of the normal deoxy spectrum. After subtraction of 55% of the Hb A spectrum, we obtained a well defined Soret band with peak at 423 nm (Fig. 1B). Spectra of deoxy heme in detergents, thought to produce the tetra-liganded form since water is eliminated as fifth ligand, show several peaks in the Soret band; thus the mutant β chains are probably not tetracoordinated. The fifth ligand is apparently not a histidine, as evidenced by the large blue shift. The spectrum is similar to that

of deoxy heme in co-solvents such as ethylene glycol or glycerol. The heme group in the deoxy mutant β chains is most likely penta-coordinated with a weak ligand, possibly a water molecule.

Fig. 2 shows the alkaline cellulose acetate electrophoresis for the mutant. Two bands are seen migrating between normal Hb A and α chains. The proportion of the more negative band greatly decreases upon addition of hemin-CN. This band is attributed to semi-Hb tetramers which are able to bind a heme, but apparently with a lower affinity than normal chains.

Oxygen binding curves at equilibrium could not be performed due to the instability of the oxygenated form of the mutant rHb. The CO bimolecular kinetics for the mutant were measured before and after addition of heme-CO, in order to measure the functional properties of tetramers with 4 hemes. The CO bimolecular recombination kinetics of rHb βH92A are nearly monophasic with a CO association rate similar to that of R-state HbA (Fig. 3). Addition of heme-CO (up to 40% of the initial heme concentration) to saturate the globins did not change the R-like recombination kinetics. Parkurst et al. [20] have measured by the stopped flow technique the CO recombination of a semi-Hb where only the α chains of $\alpha_2\beta_2$ tetramers bind the heme; the CO kinetics were rapid with a rate similar to that of isolated α chains. Since the rate of CO recombination for the R-state Hb is faster than that measured for isolated α chains, we assume that a large fraction of mutant tetramers contain 4 hemes. Under the same experimental conditions Hb A exhibits kinetics with 30% of a slow phase characteristic of T-state tetramers (30-fold slower than the fast phase). The ratio of the two phases depends on the fraction of photodissociated heme-CO for Hb A but not for rHb βH92A. The bimolecular amplitude of the CO recombination is close to 50%, comparable to the value observed in carbonyl Hb A. This is consistent with the assumption of Hb $(CO)_4$ being in the R-quaternary structure before the flash [21,22]. Therefore we conclude that tetrameric rHb βH92A is predominantly in the R-state and binds CO without cooperativity like other natural mutants at this position [12].

To test whether the cavity created by the β(F8) H92A mutation can be filled by a proximal ligand, we added 10 mM imidazole to the mutant Hb solution. The rates and amplitudes

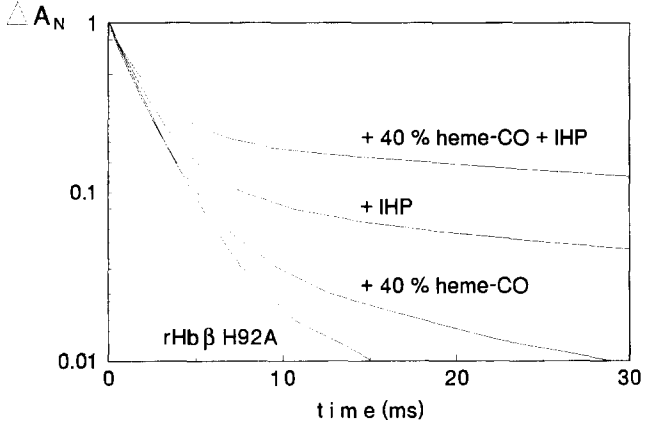

Fig. 3. Bimolecular CO recombination kinetics for Hb A and rHb βH92A at 50% photodissociation in the absence or presence of 0.2 mM IHP, plus or minus 0.1 mM L345. Additional heme-CO was added to the mutant Hb, since the spectroscopic studies indicated some heme loss. For details, see text.

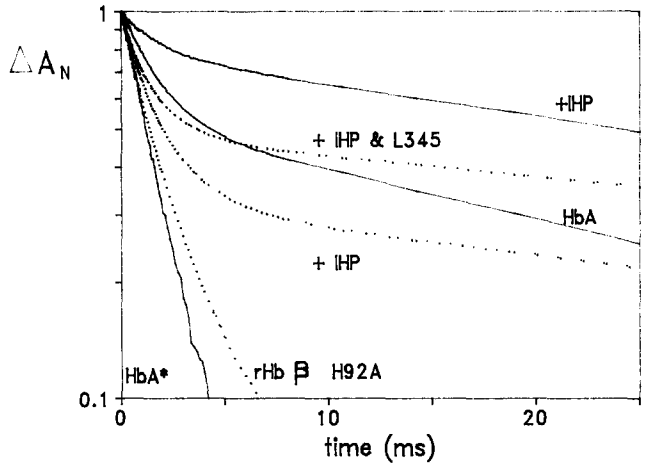

Fig. 4. Bimolecular CO recombination kinetics for Hb A and rHb βH92A. The trace labeled HbA* represents the kinetics of CO recombination to Hb A in the R-state, after 10% photodissociation. All other traces were obtained at 50% photodissociation. Solid lines are for Hb A. Dotted lines are for the rHb βH92A with and without strong effectors IHP and L345. For details, see text.

of the CO bimolecular kinetics for the mutant were not modified by addition of imidazole. It is likely that the steric repulsion induced by the F8 methyl group of alanine impedes the bond between imidazole and the heme, since this bond is allowed by the mutation (F8) His→Gly in myoglobin [14].

Addition of strong allosteric effectors inositol hexaphosphate (IHP) and L345 restores an allosteric behavior for the rHb βH92A. These two allosteric effectors have distinct binding sites, between the β chains for IHP and on the α chains for L345 [23], and their effects are additive. In these conditions the proportion of slow phase kinetics represents 30% with IHP alone, and 50% with IHP plus L345 (Fig. 3). Fig. 4 shows that the shift in the allosteric equilibrium due to the effector binding is smaller without addition of heme-CO to the mutant because of the presence of a semi-hemoglobin fraction. The proportion of rapid and slow kinetics for the tetramers with allosteric effectors is independent of the fraction photodissociation. This could be due either to a constant fraction of tetramers undergoing the allosteric transition or to a fixed fraction of tetramer in the T-state which rebinds CO with a slow rate, probably to the α subunits of the rHb βH92A. The bimolecular fraction of CO recombination increases by a few percent upon addition of effectors for normal and mutated hemoglobins indicating an R-quaternary structure before CO photodissociation [22].

While normal Hb A has a larger proportion of slow CO recombination in the presence of IHP (70% for Hb A versus 30% for the mutant), the influence of the allosteric effectors IHP and L345, for rHb βH92A, is similar. Both the mutant and Hb A (+ or − IHP) require a shift in the allosteric equilibrium of nearly an order of magnitude.

In conclusion, the substitution of the highly conserved F8 histidine by an alanine in the β subunits of Hb A results in the loss of the tetramer allosteric properties and destabilizes the low affinity quaternary structure. This mutation probably modifies to a large extent the proximal side of the heme pocket preventing the allosteric R→T transition. The loss of the proximal Fe-His bond abolishes heme-heme interactions. However, allosteric effectors can still shift the allosteric equilibrium. We

suggest that a pathway for control of the allosteric transition for this mutant still exists between the effector binding site and the $\alpha_1\beta_2$ interface, in spite of the loss of interaction between the 4 subunits via the Fe-His(F8) bond.

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