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The carbon monoxide derivative of human hemoglobin carrying the double mutation LeuB10 → Tyr and HisE7 → Gln on α and β chains probed by infrared spectroscopy

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Abstract

The fine structural properties of the distal heme pocket have been probed by infrared spectroscopy of ferrous carbon monoxy human hemoglobin mutants carrying the mutations LeuB10 → Tyr and HisE7 → Gln on the α, β, and both chains, respectively. The stretching frequency of iron-bound carbon monoxide occurs as a single broad band around 1943 cm^{-1} in both the α and the β mutated chains. Such a frequency value indicates that no direct hydrogen bonding exists between the bound CO molecule and the TyrB10 phenolic oxygen, at variance with other naturally occurring TyrB10, GlnE7 nonvertebrate hemoglobins. The rates of carbon monoxide release have been determined for the first time by a Fourier transform infrared spectroscopy stopped-flow technique that allowed us to single out the heterogeneity in the kinetics of CO release in the α and β chains for the mutated proteins and for native HbA. The rates of CO release are 15- to 20-fold faster for the mutated α or β chains with respect to the native ones consistent with the lack of distal stabilization on the iron-bound CO molecule. The present results demonstrate that residues in key topological positions (namely E7 and B10) for the distal steric control of the iron-bound ligand are not interchangeable among hemoglobins from different species.

Keywords: Human hemoglobin; Infrared spectroscopy; Ligand binding in hemoproteins; Hemoglobin mutants

In vertebrate hemoglobins and myoglobins, the side chains at the topological positions B10, E7, and E10 play a key role in the stabilization of the bound oxygen and are essential to discriminate oxygen versus other biatomic ligands such as NO and CO [1]. In particular, the distal histidine in position E7 gates the ligand entry into the pocket and stabilizes the iron-bound oxygen whereas the hydrophobic side chain of LeuB10 provides an apolar environment that favors the correct accommodation of the heme macrocycle. In contrast, in several classes of nonvertebrate hemoglobins, the positions B10, E7, and E10 can be occupied by different residues. In particular, in most nonvertebrate hemoglobins (e.g., bacterial, protozoa, yeast, fungi, and nematodes) a Tyr residue in position B10 is highly conserved whereas Gln occurs frequently in the topological position E7 [2]. These hemoglobins are characterized by a very high oxygen affinity and by a corresponding slow rate of oxygen release [2,3]. The structural basis of the high oxygen affinity and slow rate of ligand release in these hemoglobins is currently ascribed to the presence of a strong hydrogen bonding interaction of the phenolic hydroxyl of TyrB10 to the bound oxygen or to a hydrogen bonding network that involves TyrB10, GlnE7, and the bound ligand [4–9].

Unexpectedly, in HbA,[1] the introduction of TyrB10–GlnE7 motif in the distal heme pocket brings about a significant decrease in oxygen affinity and a reduced cooperativity in the absence of effects on the quaternary assembly of the protein [10]. Equilibrium ligand-binding properties of the symmetric double mutant $Hb\alpha\beta^{YQ}$

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[1] *Abbreviations used:* $Hb\alpha^{YQ}$, human hemoglobin tetramer bearing the double mutation LeuB10 → Tyr and HisE7 → Gln on the α chains; $Hb\beta^{YQ}$, same mutations on the β chains; $Hb\alpha\beta^{YQ}$, same mutations on both chains; FTIR, Fourier transform infrared spectroscopy; SVD, singular value decomposition.

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indicated that the mutated protein can still undergo the T–R quaternary transition, although with a significant depression of the K_R value (about sixfold). The kinetic counterpart of the reduced oxygen affinity has been ascribed, at least in part, to the reduced values of the second-order binding constant for O_2. A significant decrease in the second-order binding rates is also observed for other biatomic ligands and is particularly evident in the case of NO (50- to 100-fold [10]). The rationale for the decreased ligand-binding rates and for the decreased ligand affinity in $Hb\alpha\beta^{YQ}$ is provided by the 1.8 Å resolution X-ray structure of the deoxy derivative. In fact, in the deoxy conformation, TyrB10 and GlnE7 side chains appear to hinder access to the heme iron through a network of hydrogen bonds that involves a bridging water molecule between the GlnE7 nitrogen and one heme propionate [10]. Thus, a stable steric hindrance in the distal heme pocket most likely impairs free diffusion of the biatomic ligands to the iron atom contact area and depress the overall second-order binding constants.

In order to complete the kinetic characterization of the functional properties of these mutants and to unveil the fine structural properties of bound CO, the spectral properties and the kinetics of CO release in the symmetric ($Hb\alpha\beta^{YQ}$) and in the asymmetric mutants ($Hb\beta^{YQ}$ and $Hb\alpha^{YQ}$) has been investigated by FTIR spectroscopy. The results obtained are discussed in the framework of the recently obtained crystal structure of the $Hb\alpha\beta^{YQ}CO$ [11].

Materials and methods

The HbA mutants were prepared as described previously [10]. The protein was concentrated to about 4 mM with a Vivaspin II concentrator (Sartorius AG, Germany). The CO derivative was prepared with concentrated protein (200 ml) in a small vial under 1 atm of CO in the presence of 10 mM sodium dithionite (30 min equilibration at 4 °C). $^{13}C^{16}O$ and D_2O isotopes were obtained from Isotec (Miamisburg, OH).

FTIR measurements were carried out on a Magna 760 Nicolet instrument equipped with an MCT/A detector. In transmission experiments, the samples (4 mM protein in 0.1 phosphate buffer at pH 7.0) were placed on CaF_2 windows with a 50-mm Teflon spacer and 512 scans were acquired at 2 cm^{-1} resolution and two levels of zero filling. Each spectrum was baseline corrected by subtracting the spectrum of the unliganded ferrous protein. Kinetics of CO release were measured by using a thermostattable 50 μm sealed cell. The cell was directly connected to a rapid mixing Hi-Tech apparatus, equipped with 0.5-ml driving syringes. The luer port cavity (20 ml) of the sealed cell entry was adapted with an HPLC V connector that allowed mixing of the solutions before entering the cell. The flow was stopped by connecting the exit of the cell directly to the stopping syringe of the Hi-Tech apparatus. The dead time of the apparatus was about 1 s. In the CO release experiments, the hemoglobin solutions (2 mM heme), equilibrated with 1 atm CO gas, were mixed with solutions containing potassium ferricyanide (10–100 mM) and potassium cyanide (0.2 M) in 0.1 M phosphate buffer at pH 7.0. The spectra after mixing were collected at a rate of 16 interferograms per second at 2 cm^{-1} resolution. In the spectral reconstruction procedure, carried out with the OMNIC Series program (Nicolet), 64 interferograms were averaged and Fourier transformed to yield a single absorption spectrum every 4 s. The spectra of the HbCO derivatives (512 scans), measured by mixing HbCO solutions with buffer, were used as backgrounds. All experiments were carried out at 20 °C.

Singular value decomposition (SVD) analysis was carried out on the data sets (spectra vs time) by standard procedures [12] using the Matlab 5.0 software package (The Math Works Inc. Natick, MA) on an IBM PC. Briefly, the data matrix, A, is transformed in the product of three orthonormal matrices $A = USV^T$, where the U columns are the basis spectra, the V columns represent the corresponding amplitudes as a function of time, and the diagonal matrix S contains the singular values. The first two V columns (time courses) were fitted together with the first two U columns (basis spectra) by a nested loop procedure in which, in the first iteration, the two rate constants are obtained which yield the best fit to the two V columns. In the second iteration, the four coefficients of the linear combination of the three U columns (basis spectra) were obtained which yield the best fit to the observed spectra.

Results

The spectrum of the ferrous $Hb\alpha\beta^{YQ}CO$ derivative, reported in Fig. 1, displays a broad (11 cm^{-1}) asymmetric band *peaked* at 1943 cm^{-1}. An analogous spectral line shape is observed at 1898 cm^{-1} for the $^{13}C^{16}O$ isotope, confirming that no spurious bands contributes to the overall spectral profile of the CO absorption band (data not shown). The spectral profiles of the $Hb\alpha^{YQ}$ and $Hb\beta^{YQ}$ are more complex due to the superposition of the spectra relative to the wild-type and mutated chains. In these proteins the contributions of the wild-type α and β chains are clearly distinguishable as sharp peaks at 1952 ($Hb\beta^{YQ}$) and 1950.5 cm^{-1} ($Hb\alpha^{YQ}$) and are superimposable to those measured for the isolated native α and β chains [13]. In turn, the contributions of the CO absorption spectra of the mutated α or β chains, whose integrated intensity is lower than that of the native chains, can only be guessed form the skewed spectral profiles of the $Hb\beta^{YQ}$ and $Hb\alpha^{YQ}$ mutants. FTIR measurements carried out on Hb $\alpha\beta^{YQ}$ in D_2O buffered

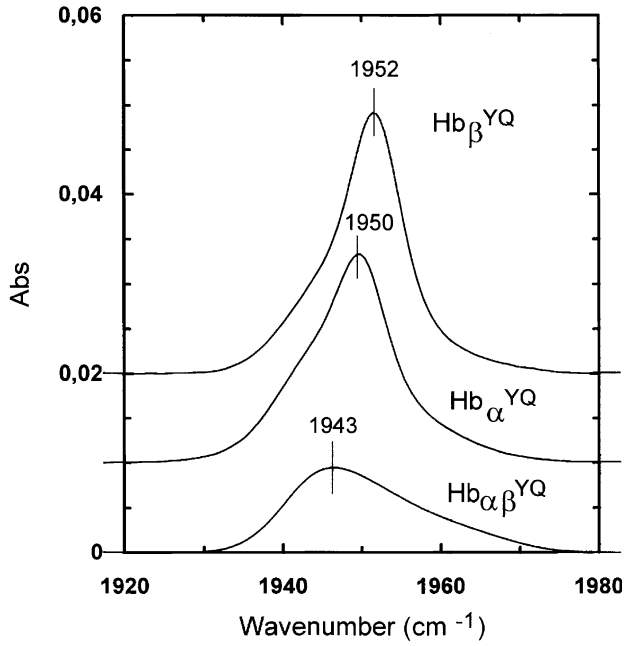

Fig. 1. Infrared absorption spectra of Hb$\alpha\beta^{YQ}$, Hbβ^{YQ}, and Hbα^{YQ}, CO-bound hemoglobins. The spectra were measured on 4 mM protein solutions equilibrated with 1 atm CO gas, in 0.1 M phosphate buffer, pH 7.0, in the presence of 10 mM sodium dithionite and at 20 °C.

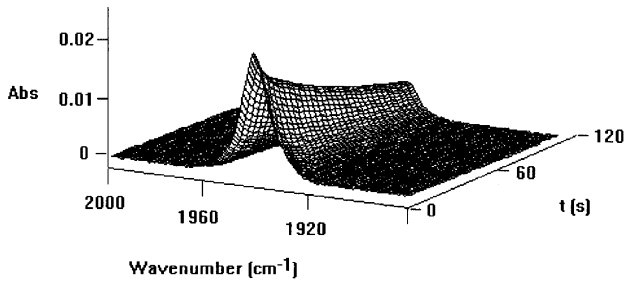

Fig. 2. Infrared absorption time course of CO release from Hbα^{YQ}. The spectrum was followed after mixing a 2 mM Hbα^{YQ} solution with a 10-fold excess potassium ferricyanide solution containing 0.1 M potassium cyanide in 0.25 M phosphate buffer at pH 7.0. Each spectrum (4 s) is the average of 64 acquisitions at 2 cm^{-1} resolution.

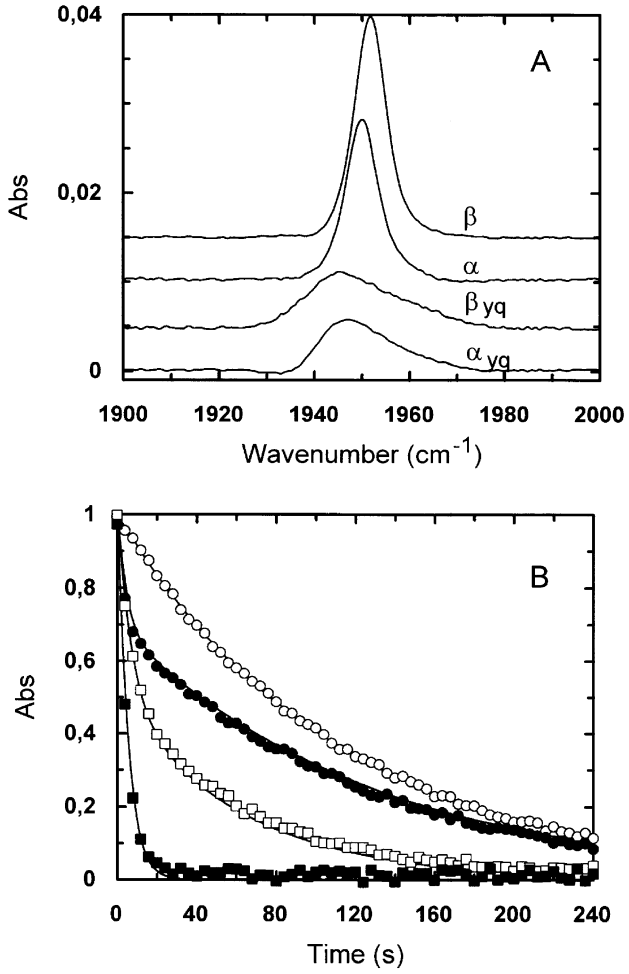

Fig. 3. Deconvoluted infrared spectra of Hb$\alpha\beta^{YQ}$, Hbβ^{YQ}, and Hbα^{YQ} hemoglobins and time courses of CO release. The infrared absorption spectra of wild-type (labeled as α and β) and mutated (labeled as α^{YQ} and β^{YQ}) α and β chains within Hbα^{YQ} and Hbβ^{YQ} asymmetric double mutants (A) have been deconvoluted by standard SVD procedure. The time courses have been obtained from the *V* columns of the SVD matrix and normalized to unity (B). The kinetic records have been fitted to single exponentials in the case of Hb$\alpha\beta^{YQ}$ (●) and to double exponentials in the case of Hbα^{YQ} (■), Hbβ^{YQ} (□), and native HbA (○).

solutions did not reveal a significant shift of the peak frequency of the CO infrared band (data not shown).

Kinetics of CO release from the mutants were measured by rapid-mixing FTIR spectroscopy in parallel with native HbA. Fig. 2 shows the time course of the absorption spectra after mixing a solution of Hbα^{YQ}CO with an excess of potassium ferricyanide (50 mM) and potassium cyanide (0.1 M). The time course is strongly biphasic with a fast phase characterized by a half-time of about 15 s and a slow phase with a half time of 80 s.

Simple inspection of the spectral changes of Fig. 2 indicates clearly that the two phases can be assigned to the CO release from the mutated α chain (fast phase) and from the wild-type β chain (slow phase). A similar profile has been obtained for the Hbβ^{YQ} mutant. SVD analysis (Fig. 3) allowed a complete deconvolution of the spectral contributions and of the time courses of the CO release relative to each chain. In turn, measurements carried out on the double mutant Hb$\alpha\beta^{YQ}$ indicated a fast overall ligand release (see Fig. 3B and Table 1), ending within 40 s after mixing. Such a rapid decay did not allow the recording of a sufficient number of spectra necessary for SVD analysis. In the case of native HbA, deconvolution of the two separate kinetic processes relative to the CO release from α and β chains could only be obtained by biasing the fitting procedure with the introduction of the deconvoluted spectra of the wild-type α and β chains obtained from the SVD analysis of

Table 1
CO dissociation rate constants for native HbA and for Hbα^{YQ}, Hbβ^{YQ}, and Hbαβ^{YQ} mutants as measured by rapid mixing FTIR spectroscopy

Protein	$K_{off}\alpha$ (s^{-1})	$k_{off}\alpha$ (s^{-1})	k_{off} (overall)
HbA	0.014 ± 0.006	0.007 ± 0.004	0.009 ± 10.003
Hbα^{YQ}	0.26 ± 0.075	0.008 ± 0.003	—
Hbβ^{YQ}	0.016 ± 0.006	0.14 ± 0.062	—
Hbαβ^{YQ}	—	—	0.19 ± 0.065

Note: All experiments were carried out in 0.1 M phosphate buffer at pH 7.0 and 26 °C.

the asymmetric hybrids. Although the degrees of freedom of fitting procedure are reduced, reasonable estimates have been obtained for the rates of CO release from each chain. In Fig. 3, the SVD averaged time courses relative to the CO release from the native and mutated chains are reported together with the SVD deconvoluted spectra of each mutated chain (obtained from the Hbβ^{YQ} and Hbα^{YQ}CO release experiments). Values of the first-order rate constants for CO release obtained from a two exponential fitting procedure are collected in Table 1.

Discussion

In the present study, the CO infrared absorption spectra of symmetrically (Hbαβ^{YQ}) and asymmetrically (Hbα^{YQ} and Hbβ^{YQ}) doubly mutated human hemoglobins have been measured and the contribution of either chain to the overall absorption spectrum has been singled out. The results obtained indicate that the effect of the LeuB10 → Tyr and HisE7 → Gln mutations on the CO infrared absorption spectrum is similar in both chains. In particular, as shown in Figs. 1 and 3 the CO stretching bands are broad and asymmetric and are peaked around 1942–1943 cm^{-1}. The line shapes and frequencies of the CO stretching bands imply that in both chains no direct hydrogen bonding occurs between TyrB10 and the iron-bound CO. Moreover, the insensitivity of the spectra to hydrogen/deuterium exchange provides evidence against the participation of a water molecule to the distal coordination of the bound CO molecule. The infrared absorption data can be discussed in the light of the recently obtained X-ray structures of the deoxy and partially liganded Hbαβ^{YQ} that contains iron-bound CO in the β chains only [6–11]. First of all it should be stressed that the partially liganded structure pertains to a T conformation whereas the infrared spectra are representative of a fully R state. Nevertheless, the distal pocket stereochemistry, as observed in the crystal structure of the T-state ligated β chains, offers several interesting points for discussion (see Fig. 4). The presence of multiple conformations of TyrB10 indicates that the phenolic ring possesses a considerable degree of freedom within the pocket in the liganded derivatives that contrasts with the rigid stereochemistry observed in the unliganded species where the phenol hydroxyl is captured in a network of hydrogen bonds involving the GlnE7 amino group, a water molecule, and one heme propionate [10]. It is somewhat surprising that, given the observed rotameric flexibility of the phenol moiety, none of the possible conformations of the tyrosine are capable of establishing an energetically favorable hydrogen-bonding interaction with the CO molecule. In fact, in the nearest ligand–TyrB10 conformer (populated for about 50% in the β1 subunit) the hydroxyl oxygen is located at 2.5 Å from the CO oxygen atom but does not exhibit the correct geometry for an hydrogen-bonding interaction (Fig. 4). In fact, in all Tyr-mediated hydrogen-bonding interactions (included TyrB10–O_2 hydrogen bonding in *Ascaris suum* Hb [8]) the phenolic O–H is preferentially oriented along the direction imposed by the sp^2 oxygen acceptor (i.e., iron-bound oxygen) doublets. Such a geometry is not observed in the present structure where the phenolic oxygen lies well aside from the optimal direction with respect the nearest oxygen doublet of the iron-bound CO. Moreover, the present data in solution demonstrate that also in the R state no interaction between TyrB10 and iron-bound CO is present.

It is of interest to compare the present result with the infrared and resonance Raman spectra of several naturally occurring TyrB10–GlnE7 hemoglobins [3–7]. In these proteins the CO stretching band is often split in two discrete peaks, namely a low-frequency mode centered at about 1908–1912 cm^{-1} and a higher frequency component comprised between 1940–1960 cm^{-1}. The low-frequency peak has been attributed to the presence of a hydrogen-bonding interaction between bound CO and the phenolic hydroxyl of TyrB10 whereas the high-frequency component pertains to an "open" conformation where such an interaction is not present [5]. A great variability in the CO stretching intensity ratio between the two conformers is observed among different proteins up to *Lucina* HbII hemoglobin that exhibits a single CO stretching mode at 1960 cm^{-1} [3]. In this framework, attempts to correlate the vibrational properties of the CO-bound derivatives to the kinetics of CO release and possibly extend the correlation further to the oxygenated derivatives has proven to be a difficult task, at least in these proteins. In general, as demonstrated in a number of mutated myoglobins, the CO stretching frequencies are linearly correlated leads to ligand dissociation rates [14]. In particular, low CO stretching frequencies (and thus high Fe–CO stretching frequencies) correspond to slow rates of ligand release [14]. The presence of two discrete conformers and the observation of a single, relatively fast, CO dissociation rate in *A. suum* Hb has been explained by Das et al. [5] with a fast interconversion between a closed (low CO stretching frequency) and an

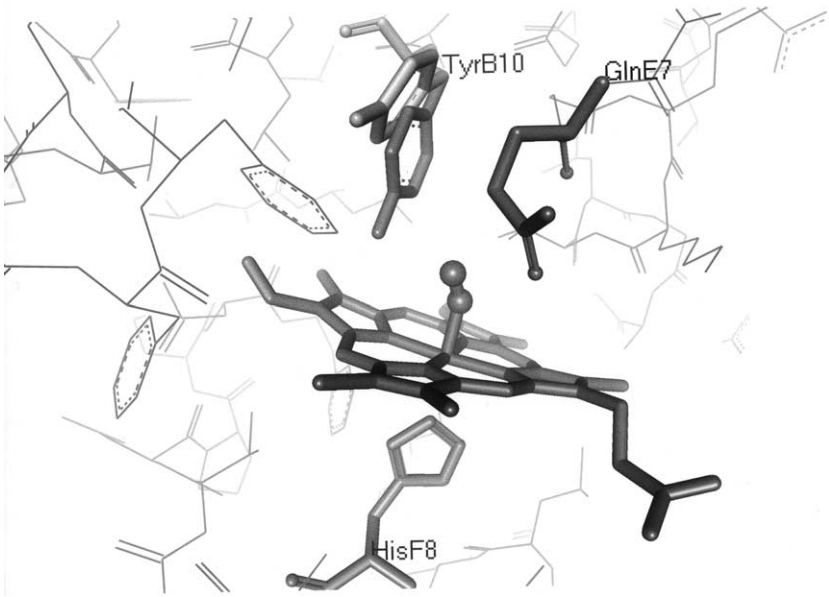

Fig. 4. Close-up view of the heme pocket of β1 chain of Hbαβ^{YQ} CO-bound derivative (pdb code 1J7Y). Amino acids B10, E7, and F8 are rendered in stick, together with heme and the iron-bound CO molecule. The two conformations of TyrB10 (dark gray and light gray) have been refined to 50% each, without residuals in the difference map [11].

open (high CO stretching frequency) conformation of the distal heme pocket. The open conformer eventually allows CO escape at a rate (0.018 s^{-1}) that is in reasonable agreement with observed CO stretching frequency (1948 cm^{-1}). Nevertheless, the correlation between stretching frequencies and rates of ligand dissociation obtained with CO cannot be directly transferred to the functionally relevant oxygenated species in that iron-bound oxygen is more polar than iron-bound CO and is likely to establish stronger interaction with the relevant TyrB10 and GlnE7 residues [5].

In this framework, the relatively fast kinetics of CO release in Hb^{YQ} hemoglobins are consistent with the absence of a hydrogen-bonding interaction with Tyr B10 and with a relatively open conformation of the heme pocket in the R state. In fact, the CO dissociation experiments point out clearly that the mutated chains are capable of 15- to 20-fold faster release of the ligand with respect to the native ones. It should be pointed out that no R–T transition occurs upon CO release in that cyanide binds rapidly to the ferric heme iron produced in the ferricyanide oxidation reaction [15]. In contrast, the rates for oxygen dissociation from the R state in Hbαβ^{YQ} indicate that oxygen release is significantly decreased in the α chains (about 10-fold) and nearly identical in the β chains with respect to the native chains [10,11]. The different effect of the mutations on the release of CO and oxygen may be interpreted according to the considerations on *A. suum* Hb reported above. It may be envisaged that the iron-bound oxygen molecule is capable of establishing an effective hydrogen-bonding interaction with the phenolic hydroxyl of TyrB10 whereas iron-bound CO is not.

As a last comment, it is worth noting that the rate of CO release is about twofold faster in the α chain than in the β chains in both the mutated and the wild-type chains within the single mutants. The functional heterogeneity in the CO release from native HbA has been discussed in detail by Samaja et al. [15] and has been shown to be a complex phenomenon in which the intrinsically faster rate of CO release of the α chains with respect to the β ones is modulated by both homotropic and heterotropic effects. Thus, the faster release of CO from the mutated α chains with respect to the mutated β chains might suggest that the intrinsically different reactivity of α and β chains depend on factors that are not uniquely linked to the distal pocket geometry but are related to different overall dynamics of the polypeptide chains.

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References

[1] B.A. Springer, S.G. Sligar, J.S. Olson, G.N. Phillips Jr., Chem. Rev. 94 (1994) 699–714.

[2] M. Bolognesi, D. Bordo, M. Rizzi, C. Tarricone, P. Ascenzi, Prog. Biophys. Mol. Biol. 69 (1997) 29–58.
[3] A.M. Gardner, L.A. Martin, P.R. Gardner, Y. Dou, J.S. Olson, J. Biol. Chem. 275 (2000) 12581–12589.
[4] E.P. Peterson, S. Huang, J. Wang, L.M. Miller, G. Vidugiris, A.P. Kloek, D.E. Goldberg, M.R. Chance, J.B. Wittenberg, J.M. Friedman, Biochemistry 36 (1997) 13110–13121.
[5] T.K. Das, M. Couture, Y. Ouellet, M. Guertin, D.L. Rousseau, Proc. Natl. Acad. Sci. USA 98 (2001) 479–484.
[6] T.K. Das, J.M. Friedman, A.P. Kloek, D.E. Goldberg, D.L. Rousseau, Biochemistry 39 (2000) 837–842.
[7] A.P. Kloek, J. Yang, F.S. Mathews, C. Frieden, C. Poyart, Proc. Natl. Acad. Sci. USA 91 (1994) 1594–1597.
[8] M. Couture, T.K. Das, H.C. Lee, J. Peisach, D.L. Rousseau, B.A. Wittenberg, D.L. Rousseau, M. Guertin, J. Biol. Chem. 274 (1999) 6898–6910.
[9] M. Couture, T.K. Das, P.Y. Savard, Y. Ouellet, J.B. Wittenberg, B.A. Wittenberg, D.L. Rousseau, M. Guertin, Eur. J. Biochem. 267 (2000) 4770–4780.
[10] A.E. Miele, S. Santanchè, C. Travaglini-Allocatelli, B. Vallone, M. Brunori, A. Bellelli, J. Mol. Biol. 290 (1999) 515–524.
[11] A.E. Miele, F. Draghi, A. Arcovito, A. Bellelli, M. Brunori, C. Traveglini-Allocatelli, B. Vallone, Biochemistry 40 (2001) 14449–14458.
[12] E.H. Henry, J. Hofrichter, Methods Enzymol. 210 (1992) 129–192.
[13] W.T. Potter, J.H. Hazzard, S. Kawanishi, W.S. Caughey, Biochem. Biophys. Res. Commun. 116 (1983) 719–725.
[14] T. Li, M.L. Quillin, G.N. Phillips, J.S. Olson, Biochemistry 33 (1994) 1433–1446.
[15] M. Samaja, E. Rovida, M. Niggleler, M. Perrella, L. Rossi-Bernardi, J. Biol. Chem. 262 (1987) 4528–4533.