Quaternary Structure Dependence of Kinetic Hole Burning and Conformational Substates Interconversion in Hemoglobin†

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ABSTRACT: Using a sol-gel encapsulation technique, we have prepared samples of CO saturated human adult hemoglobin locked in the R or T quaternary conformation. We report time-resolved spectra of these samples in the Soret region following flash photolysis, in the time interval ranging from 250 ns to 200 ms and in the temperature interval of 100−170 K. A suitable analysis of the measured difference spectra enables us to obtain the spectral contribution of deoxyHb and HbCO molecules as a function of time and/or of the fraction N(*t*) of deoxyHb molecules. In our experimental time window geminate CO rebinding to hemoglobin in the T quaternary conformation is about 2 orders of magnitude slower than to hemoglobin in the R conformation: this suggests that the barrier distribution for the CO rebinding, $g(H)$, depends strongly on the protein quaternary structure. In our temperature interval, spectral shifts due to kinetic hole burning (KHB) are present: for HbCO the KHB effect is large in the R conformation and small in the T conformation. For deoxyHb the opposite is true. We attribute the observed behavior to the effect of interconversion between the relevant substates. This effect is stronger for HbCO molecules in the T conformation and for deoxyHb molecules in the R conformation; it confirms the quaternary structure dependence of the hemoglobin energy landscape and suggests enhanced dynamics of ligation intermediate species such as T-state HbCO or R-state deoxyHb.

Studies on protein dynamics are important since dynamics is crucial for protein function (*1*). Myoglobin (Mb)[1] is the protein most commonly used for such kind of studies; works on the structural dynamics of Mb (*2*, *3*), besides contributing to the understanding of the structure−dynamics−function relationships in this protein, have provided some general concepts in protein dynamics. In fact, concepts such as energy landscape, taxonomic conformational substates, or statistical conformational substates have been highlighted essentially from studies on myoglobin (*4*−*8*).

For hemoglobin (Hb), studies on dynamic properties are complicated by quaternary structure relaxation (*9*) and by $\alpha-\beta$ heterogeneity (*10*). Nevertheless, such kind of studies are important since the characterization of the dynamic properties of both equilibrium species (e.g., fully ligand free and fully liganded Hb) and nonequilibrium intermediates (e.g., liganded Hb in T quaternary conformation or unliganded Hb in R quaternary conformation) is of paramount importance for the full understanding of the allosteric properties of this molecule.

Recently, it has been shown that encapsulation of Hb in porous silica hydrogels obtained from tetramethyl orthosilicate (TMOS) with the sol-gel method is a useful approach to stabilize intermediates otherwise not easily accessible. In fact, the gel matrix traps the protein and inhibits (or greatly slows down) the quaternary structure transition while allowing diffusion of solvent and small solute molecules (like salts or ligand molecules such as O_2 or CO) (*11*−*14*). In this way, when unliganded or liganded hemoglobin is encapsulated, the quaternary T and R conformations, respectively, are maintained even when the ligation state of the heme is changed. Sol-gel encapsulation is therefore a promising approach toward the characterization of the dynamic properties and of the energy landscape of both equilibrium and intermediate species of hemoglobin (*15*, *16*).

Ligand rebinding kinetics has been extensively used to probe protein dynamics. In a typical experiment, a fully CO saturated protein sample is photodissociated with a short light pulse, and CO rebinding is followed spectroscopically as a function of time. In this time-resolved experiment, two (at least two) classes of phenomena are usually observed: geminate rebinding and solvent phase rebinding. Geminate rebinding is a unimolecular reaction in which the photolyzed CO rebinds to the heme directly from various docking sites within the heme pocket. Solvent phase rebinding is a bimolecular reaction in which the CO molecule binds to the heme from the solvent after diffusing through the protein. At temperatures lower than 180 K, ligand escape from the heme pocket to the solvent, and vice versa, diffusion of

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[1] Abbreviations: deoxyHb, deoxygenated human hemoglobin; HbCO, carbonmonoxy human hemoglobin; HbO_2, oxygenated human hemoglobin; KHB, kinetic hole burning; DHF, dynamic hole filling; Mb, myoglobin; Hb, hemoglobin; TMOS, tetramethyl orthosilicate; OMA, optical multichannel analyzer.

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ligand from the solvent to the heme, are inhibited, so that only geminate rebinding is observed.

In this context, spectrally resolved studies have yielded relevant information. In particular, studies on the near-infrared band III (which is observed only for the unliganded derivatives of myoglobin and hemoglobin) have shown the presence of kinetic hole burning (KHB)—i.e., a spectral shift during rebinding—that is indicative of an heterogeneous system (*17*−*20*).

Studies on band III are complicated by the weak extinction coefficient of this band (∼400 M^{-1} cm^{-1} at room temperature) that requires use of rather concentrated samples. Nevertheless, they are greatly simplified by the fact that CO saturated proteins do not absorb in the band III spectral region. This fact, while greatly simplifying the spectral analysis, enables us to investigate only the unliganded molecules and prevents us from following the liganded ones.

Analogous studies on the Soret band spectral region, although requiring much less concentrated samples, are complicated by the need of separating the overlapping contributions of deoxygenated and CO ligated proteins. Still, such studies are highly desirable since from them information on both the deoxygenated and the CO ligated components can be obtained.

In this paper, we present a spectrally (Soret region) and time-resolved study of the CO rebinding kinetics to hemoglobin encapsulated in porous silica hydrogels obtained with the sol-gel method. Two samples have been studied in the temperature range of 100−170 K:

—in the first one, the equilibrium form of HbCO is encapsulated. This sample is HbCO in R quaternary conformation

—in the second one, deoxyHb is first encapsulated, and only after the gel is formed and aged, the sample is equilibrated with CO, thus leading to a sample of HbCO in T quaternary conformation.

The aim of the work is to characterize the energy landscape of deoxyHb and HbCO through the KHB effect and to investigate its dependence on quaternary conformation. The novelty of the approach lies on the combined use of the sol-gel encapsulation technique and of the Soret band spectral deconvolution developed at our laboratory (*21*): this enables us to study spectral shifts, KHB, and relaxations of both the deoxy and CO components in the same experiment. Therefore, information is obtained not only on the equilibrium deoxyHb and HbCO but also on the otherwise barely accessible intermediate species R-state deoxyHb and T-state HbCO.

MATERIALS AND METHODS

Sample Preparation. Human hemoglobin was prepared and stored following a standard procedure previously described (*22*). HbO_2 from concentrated stocks (∼12 wt %) was diluted in suitable water−glycerol−buffer solutions to obtain the final hemoglobin concentration (∼8 μM in heme) in 75% v/v glycerol and 0.03 M phosphate buffer pH 7.

The silica sol was synthesized from TMOS (Merck) according to the method of Ellerby et al. (*23*) with some modifications. A mixture of 60% TMOS, 38% deionized water, and 2% HCl 0.04 M was sonicated for 20 min in an ice bath and then mixed in a 2:3 proportion with the Hb solution. The gel is formed within several minutes. From this general procedure, two different kinds of Hb gels have been prepared for this study.

Sample 1: R-State HbCO. An HbO_2 solution was equilibrated with CO for ∼1 h; a slight excess of sodium dithionite was also added to have a fully reduced sample. The silica gel obtained from this HbCO solution was soaked with a protective solution (75% v/v glycerol, 0.03 M phosphate buffer, pH 7) and left to age overnight at 4 °C in a CO atmosphere. Sample 1 is therefore a gel of HbCO in the R quaternary conformation.

Sample 2: T-State HbCO. A deoxyHb solution was obtained from HbO_2 by deoxygenation with N_2 gas followed by addition of ∼5 mM sodium dithionite. The silica gel obtained from this deoxyHb solution was soaked with protective solution containing 5 mM sodium dithionite and left to age overnight at 4 °C in a N_2 atmosphere. At this stage, the gel contains Hb in the T quaternary conformation and in the deoxy ligation state. The gel (still in the protective solution) was then exposed to CO by bubbling the protective solution for ∼4 h with opportunely humidified CO gas. As previously shown (*11*, *15*), this procedure leads to a gel in which Hb is still in the T quaternary conformation but in the CO ligation state.

Experimental Apparatus. The hemoglobin gel was placed in a 4 mm thick sample holder, sealed, and mounted on a Graseby-Specac storage cryostat (P/N 21525). The temperature was maintained constant within 1 K in the 100−170 K range by a standard Specac temperature controller.

Photolysis of the ligand was achieved by the frequency-doubled output (532 nm) of a Nd:YAG pulsed laser (pulse width ∼5 ns) at sufficiently low frequencies to allow virtually full rebinding between consecutive flashes. A white continuum measuring light was dispersed by a Jobin Yvon HR320 spectrograph, using a medium resolution grating (spectral resolution = 0.2 nm, corresponding to 11 cm^{-1} at 425 nm). The absorption change because of the formation of ligand-free Hb (Hb*) was measured by an optical multichannel analyzer (OMA) setup consisting of a gated intensified photodiode array (IRY-512), a gate pulse generator (PG-10), and a detector controller (ST-120) from Princeton Instruments. A 60 nm spectral range covering the Soret band was imaged on the 512 element diode array. Difference spectra after flash photolysis were collected by accumulating 100 scans per spectrum at 10 time delays per decade that have been selected in a logarithmically equidistant fashion from 250 ns to 200 ms. The timing of the experiment was controlled by home-built electronics.

Singular value decomposition (SVD) of the data matrix consisting of consecutive difference spectra at selected temperatures yielded orthonormal spectral and kinetic eigenvectors and corresponding singular values. Comparison of the singular values with the noise content of the data and analysis of the autocorrelation of the eigenvectors allow the estimation of the rank of the data matrices. The gradual, small shift of the observed positive and negative bands in the difference spectra, because of kinetic hole burning, would theoretically render the rank of the data matrix equal to the number of columns. However, the magnitude of the shift is small enough to permit reconstruction of the data within noise by using the first two or three SVD components only.

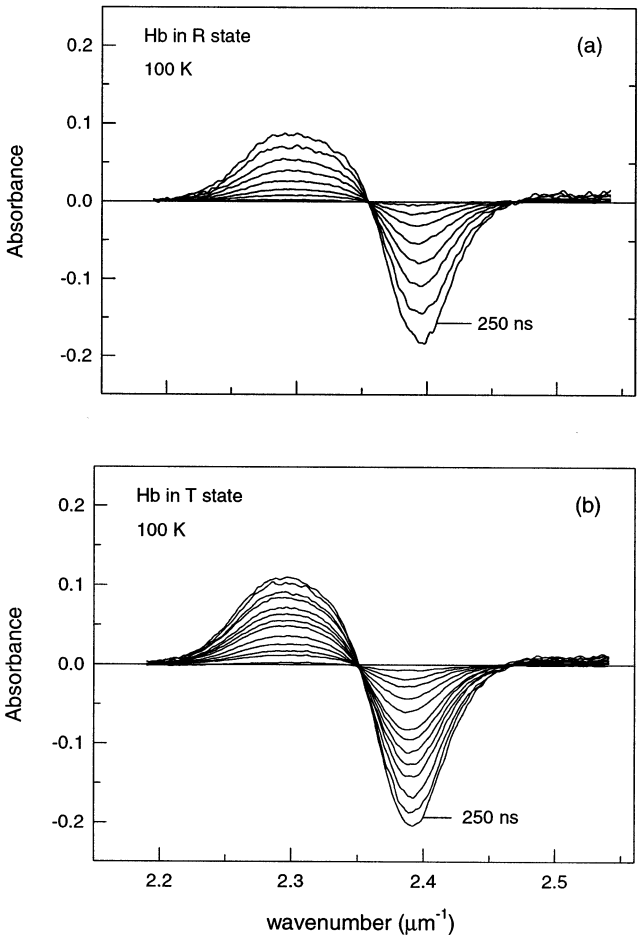

FIGURE 1: Time-resolved absorption difference spectra measured after photodissociation of adult human HbCO at $T = 100$ K. (a) R-state hemoglobin and (b) T-state hemoglobin. Positive signals arise from the deoxy-component and negative signals from the CO-component.

Typical results of our experiment are reported in Figure 1, which shows difference spectra measured at various times after photolysis at $T = 100$ K for the gel of Hb in R quaternary conformation (Figure 1a) and for the gel of Hb in T quaternary conformation (Figure 1b).

Spectral Analysis. The output of the OMA is the quantity $\Delta A(\nu,t)$ (i.e., the absorption difference spectrum between the sample at time t after photolysis and the sample before photolysis)

$$\Delta A(\nu,t) = A(\nu,t) - A_{CO}(\nu) \tag{1}$$

The absorption spectrum of the sample at time t after photolysis, $A(\nu,t)$, is a mixture of the spectral contributions arising from both the liganded and the unliganded species:

$$A(\nu,t) = A_{deoxy}(\nu,t) + A_{CO}(\nu,t) \tag{2}$$

where $A_{deoxy}(\nu,t)$ and $A_{CO}(\nu,t)$ are the spectral contributions of proteins that have not yet rebound or have already rebound the CO ligand at time t after photolysis, respectively. Therefore,

$$\Delta A(\nu,t) = A_{deoxy}(\nu,t) - A^*_{CO}(\nu,t) \tag{3}$$

where

$$A^*_{CO}(\nu,t) = A_{CO}(\nu) - A_{CO}(\nu,t) \tag{4}$$

To fit the measured difference spectra $\Delta A(\nu,t)$ we assume for $A_{deoxy}(\nu,t)$ and for $A^*_{CO}(\nu,t)$ the following analytical expression (*21*):

$$A(\nu) = M^2\nu[L(\nu)\otimes G(\nu)\otimes P(\nu)] \tag{5}$$

where M is a constant proportional to the matrix element of the electric dipole moment, and the $\otimes$ symbol stands for the convolution operator. The first term of the convolution, $L(\nu)$, is equal to

$$L(\nu) = \sum_{m_1\cdots m_{N_h}}^{\infty}\left[\prod_h^{N_h}\frac{S_h^{m_h}e^{-S_h}}{m_h!}\right]\frac{\Gamma}{[\nu - \nu_0(T) - \sum_h^{N_h} m_h\nu_h]^2 + \Gamma^2} \tag{6}$$

where

—N_h is the number of high-frequency modes (normal modes for which $h\nu \gg kT$) vibronically coupled to the electronic transition

—S_h is the linear coupling constant between the electronic transition and the h-th high frequency (ν_h) normal mode

—ν_0 is the frequency of the purely electronic transition

—Γ is the homogeneous (Lorentzian) width of the band

The second term, $G(\nu)$, describes the coupling of the electronic transition with a bath of soft modes ($h\nu$ of the same order of magnitude as kT, or smaller than kT). It can be shown that $G(\nu)$, in the so-called short times approximation (*24*), is a Gaussian

$$G(\nu) = \frac{1}{\sigma(T)(2\pi)^{1/2}}e^{-\nu^2/2\sigma^2(T)} \tag{7}$$

The third term, $P(\nu)$, describes the spectral heterogeneity of the system: molecules in different conformational substates may correspond to different frequencies of the considered electronic transition. In the hypothesis of a quadratic coupling between the iron out-of-plane coordinate Q and the Soret band peak frequency ν_0, it has been shown (*25*, *26*) that, for heme proteins in unliganded state, the spectral heterogeneity can be well-approximated by an expression first introduced by Champion and co-workers (*19*, *25*):

$$P(\nu) = \frac{\exp\left[-\frac{(\sqrt{\nu - \nu_{00}} + Q_0\sqrt{b})^2}{2b\delta^2}\right] + \exp\left[-\frac{(\sqrt{\nu - \nu_{00}} - Q_0\sqrt{b})^2}{2b\delta^2}\right]}{2\delta\sqrt{2\pi b(\nu - \nu_{00})}} \tag{8}$$

This is the expression that we have assumed in eq 5 to analyze the deoxy-component, $A_{deoxy}(\nu,t)$, of the difference spectra.

For heme proteins in liganded state, instead, the $P(\nu)$ term is a further Gaussian that does not affect the overall band shape predicted by eq 5, except for the addition of a temperature independent term to the Gaussian width of the band. A Gaussian $P(\nu)$ has thus been assumed in eq 5 for

Table 1: Values of the Parameters[a] Maintained Constant during the Fittings of the Hb Difference Spectra Measured after Photolysis

	S_{370}	S_{200}	S_{674}	S_{1357}	Γ (cm^{-1})	$Q_0b^{1/2}$ (μm^{-1})	$\delta b^{1/2}$ (μm^{-1})
deoxyHb in R-state	0.30	0.07	0.30	0.07	175	0.180	0.200
deoxyHb in T-state	0.30	0.07	0.27	0.07	175	0.180	0.200
HbCO in R-state[b]	0.07		0.09	0.11	200		
HbCO in T-state[b]	0.07		0.09	0.11	200		

[a] Parameters S_h are indexed as follows: h is the wavenumber (in cm^{-1}) of the corresponding high-frequency mode. [b] For HbCO, S_h values refer to high-frequency modes at 350, 676, and 1374 cm^{-1}, respectively.

the deconvolution of the CO-component, $A^*_{CO}(\nu,t)$, of the difference spectra. $A^*_{CO}(\nu,t)$ is, actually, the difference of two convoluted spectral functions (eq 4), and it is not obvious that it should also have the analytical form described by eq 5. Since, however, the variation of the Gaussian factor in the convolution as a function of time during rebinding is expected to be small (and indeed, almost time independent values of parameter σ have been obtained from the fits), the shape of $A_{CO}(\nu,t)$ and $A_{CO}(\nu)$ should be rather similar. This is expected to result in their difference, $A^*_{CO}(\nu,t)$, to have a similar shape with varying amplitude as rebinding proceeds.[2]

The fitting procedure optimizes the following parameters:

—integrated absorbance: $M_{deoxy}(t)$, $M_{CO}(t)$

—peak frequency: $\nu_{deoxy}(t)$, $\nu_{CO}(t)$

—Gaussian width: $\sigma_{deoxy}(t)$, $\sigma_{CO}(t)$

Values of the other parameters (i.e., Γ, S_h, ν_h, $Q_0b^{1/2}$, and $\delta b^{1/2}$) are reported in Table 1: they have been determined from equilibrium spectral measurements (27) and have been maintained constant in the fittings. This is justified by the fact that spectral variations observed involve essentially intensity variations, peak shifts, and band broadenings; moreover, it is justified a posteriori by the excellent quality of the fits. A typical fit of a difference absorption spectrum is reported in Figure 2. The fitting quality is excellent, as judged also from the residuals reported in the upper panel.

RESULTS AND DISCUSSION

From a suitable normalization of the $M_{CO}(t)$ parameter, we have calculated the fraction N(t) of proteins that have not rebound a ligand at time t after photolysis. In Figure 3, we report a typical result for the time dependence of N(t): the kinetic traces measured for the two hemoglobin samples and for a sample of sperm whale myoglobin (swMb) at T = 100 K are reported in a lin−log plot. It is evident that the rebinding kinetics are not exponential in time and that the Hb molecules in the R quaternary conformation rebind the CO ligand about 2 orders of magnitude faster than the Hb molecules in T quaternary conformation and about 4 orders of magnitude faster than the Mb molecules at N(t) values higher than 10%.

Although both peak frequencies and Gaussian widths are obtained from our fits, σ^2 values are subject to rather large errors essentially due to the smallness of the signal. Within the experimental error, constant values of σ_{CO} and of σ_{deoxy} are obtained; for this reason, in the following, we will discuss only data relative to peak frequency shifts.

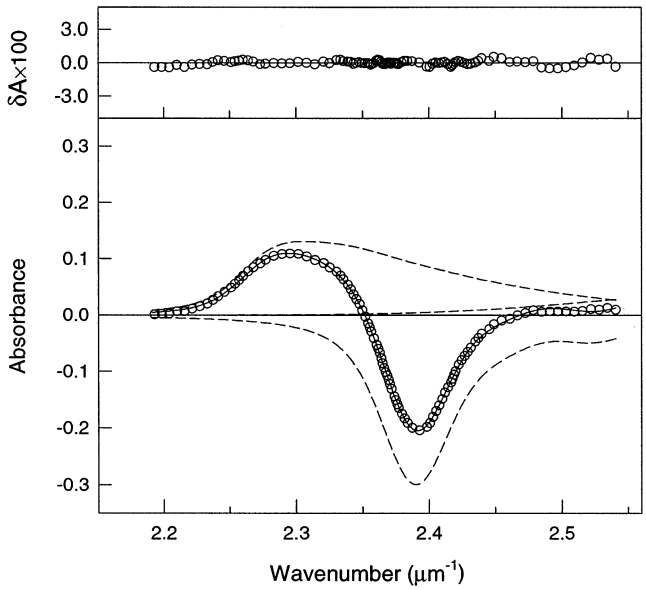

FIGURE 2: Typical fit of a difference spectrum obtained with the analytical expression given in the text. Open circles are the experimental points; the dashed lines represent, respectively, the deoxy-component, the CO-component, and a further Gaussian extrapolation that takes into account the contribution of the band N at higher frequency. The solid line is the overall fitted profile of the band. The residuals of the fit are reported in the upper panel in an expanded scale. T = 140 K, t = 250 ns, T-state Hb.

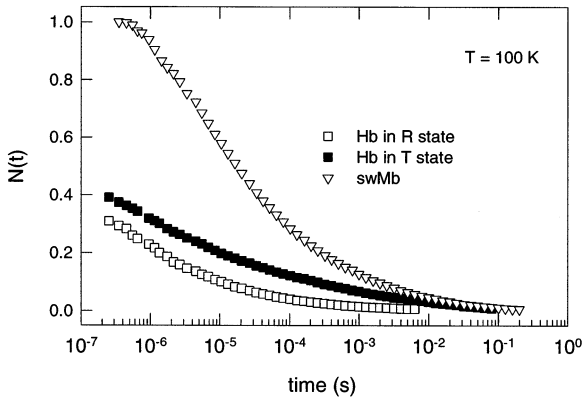

FIGURE 3: Comparison of the rebinding kinetics of carbon monoxide after photodissociation for three different samples at T = 100 K: R-state Hb gel (empty squares), T-state Hb gel (black squares), and swMb in 75% glycerol/water (empty triangles). N(t) is the fraction of proteins that do not have a ligand bound at time t after photodissociation.

Figure 4 shows the time dependence of ν_{deoxy} (Figure 4a) and ν_{CO} (Figure 4b) for the two Hb samples at T = 100 K. Data show that, during the rebinding kinetics, the deoxy-component of the Soret band shifts to the blue, while the CO-component shifts to the red; the extent of these spectral shifts depends strongly on the quaternary state of the Hb molecules. This behavior is remarkably different from the Mb one: indeed, in the case of myoglobin, both the deoxy and the CO spectral components shift to the blue during ligand rebinding (ref *28*; Levantino et al., unpublished results). Note, however, that for HbCO the fitted function is $A^*_{CO}(\nu,t)$, not the CO-component of the absolute spectrum.

[2] We note that although the quality of the resulting fits obtained is excellent (see Figure 2), this approximation may lead to underestimated peak frequency shifts for the CO-component of the spectra. We stress that this does not affect in any way the results obtained for the deoxy-component, which is almost entirely determined by the red edge of the spectrum.

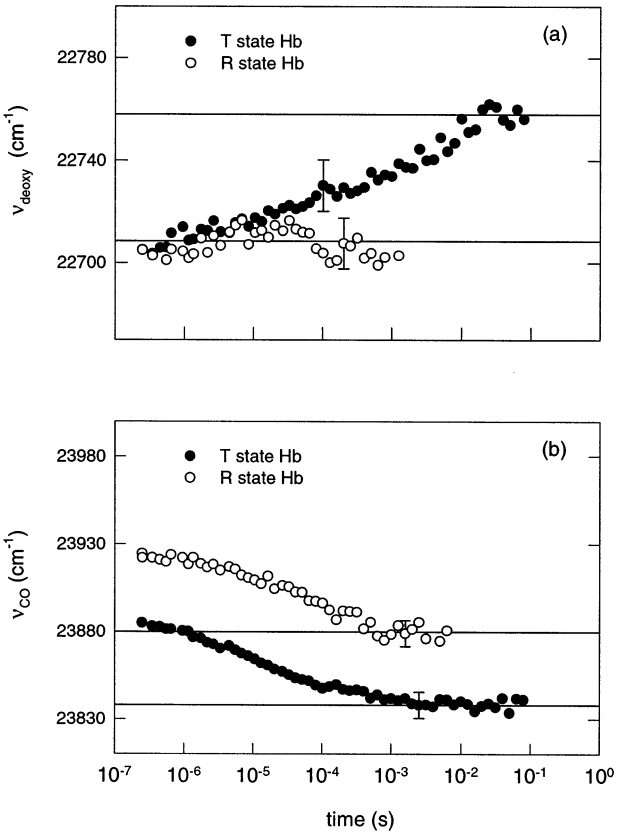

FIGURE 4: Time evolution at $T = 100$ K of the peak frequency of both (a) the deoxy-component and (b) the CO-component of the Soret band following photolysis of HbCO. Empty symbols refer to T-state Hb and closed symbols refer to R-state Hb. Typical error bars are shown.

Since, however, the peak frequency red shift of $A^*_{CO}(\nu,t)$ reflects the behavior of the CO-component of the absolute spectrum, then in HbCO the correlation between the peak frequency of the absorption band and the enthalpy for rebinding is different from the one found in MbCO. While the MbCO molecules that rebind faster are those that contribute mainly to the red side (causing a blue shift of the CO-component), the HbCO molecules that rebind faster are those that contribute mainly to the blue side (causing a red shift of the CO-component). This, in turn, implies a totally different interaction of the bound CO with the heme pocket in MbCO and HbCO. Alternatively, the difference $A^*_{CO}(\nu,t)$ is expected to have the same peak frequency as $A_{CO}(\nu)$ at $t = 0$ (i.e., at full photolysis) and may have the same peak frequency at $t \rightarrow \infty$. The time course of $A^*_{CO}(\nu,t)$ could, therefore, be a rapid blue shift and its reverse at later times. With our time resolution, we may only observe the second phase (i.e., the return to the peak frequency of $A_{CO}(\nu)$ appearing as a red shift) in accordance with the fact that N(t) is already as low as 40 and 32% at the shortest time delay of the experiment for the T- and R-states, respectively. In this second case, the coupling between the enthalpy distribution and the spectral properties would be essentially similar in Mb and Hb, and the observed red shift would be caused by the so-called dynamic hole filling (DHF). Although a detailed analysis of $\Delta\nu_{CO}$ gives some hints (see below), further experiments with improved time resolution are needed to definitely discriminate between the above two alternative hypotheses.

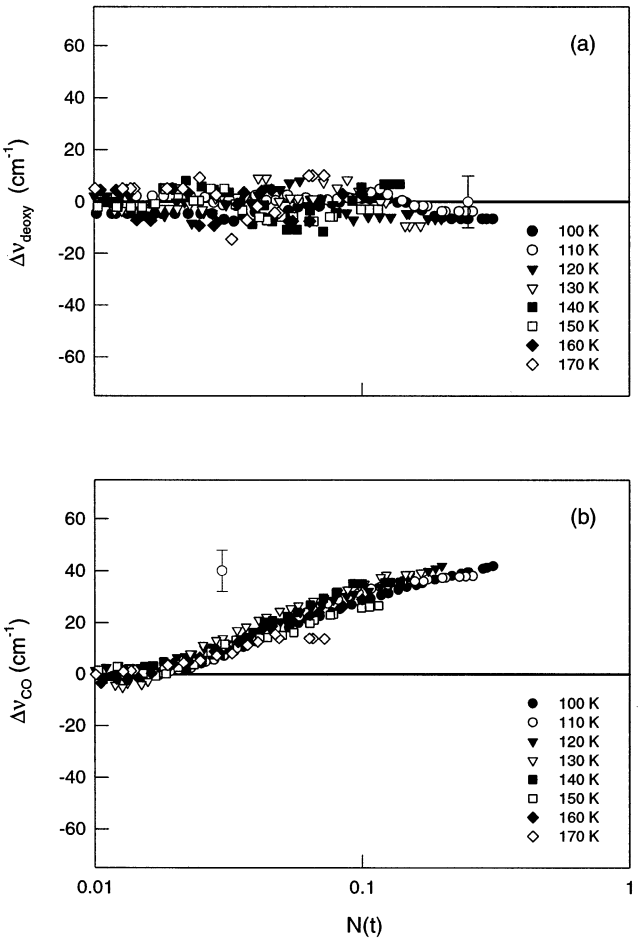

FIGURE 5: Peak frequency shift of (a) the deoxy-component and (b) the CO-component of the Soret band vs N(t) for R-state Hb in the temperature range of 100−170 K. A typical error bar (±10 cm^{-1} for $\Delta\nu_{deoxy}$; ±7.5 cm^{-1} for $\Delta\nu_{CO}$) is reported.

To evaluate the presence of KHB, we plot the peak frequency shift as a function of N(t) (*29*, *30*). Peak frequency shifts are defined as $\Delta\nu(t) = \nu(t) - \nu(\infty)$ (i.e., as the difference between the peak frequency observed at time t after photolysis and that observed at a time after photolysis sufficiently long to consider the rebinding process essentially complete). In practice, we have determined $\nu(\infty)$ as an average over the points at which N(t) < 1% (solid lines in Figure 4). Such plots are reported in Figures 5 and 6 for the R- and T-state Hb samples, respectively.

Figure 5b shows that, for HbCO in R quaternary conformation, all the $\Delta\nu_{CO}$ values fall on the same curve, independent of the temperature. Such a universal behavior has been previously obtained for Mb at low temperatures (*29*, *30*); it is a strong evidence of the absence of conformational relaxation since any conformational relaxation of the system would imply a temperature dependent deviation from the universal (i.e., purely KHB) behavior (*29*, *30*). We can conclude that the spectral shifts observed in this temperature range are essentially because of KHB. The universal behavior observed for $\Delta\nu_{CO}$ of the R-state HbCO, in turn, argues against the possibility that the observed red shift is due to dynamic hole filling: indeed, substates interconversion responsible for DHF (*20*) could hardly generate a universal behavior.

For Hb in R quaternary conformation, no $\Delta\nu_{deoxy}$ has been observed, within our experimental error, between 100 and

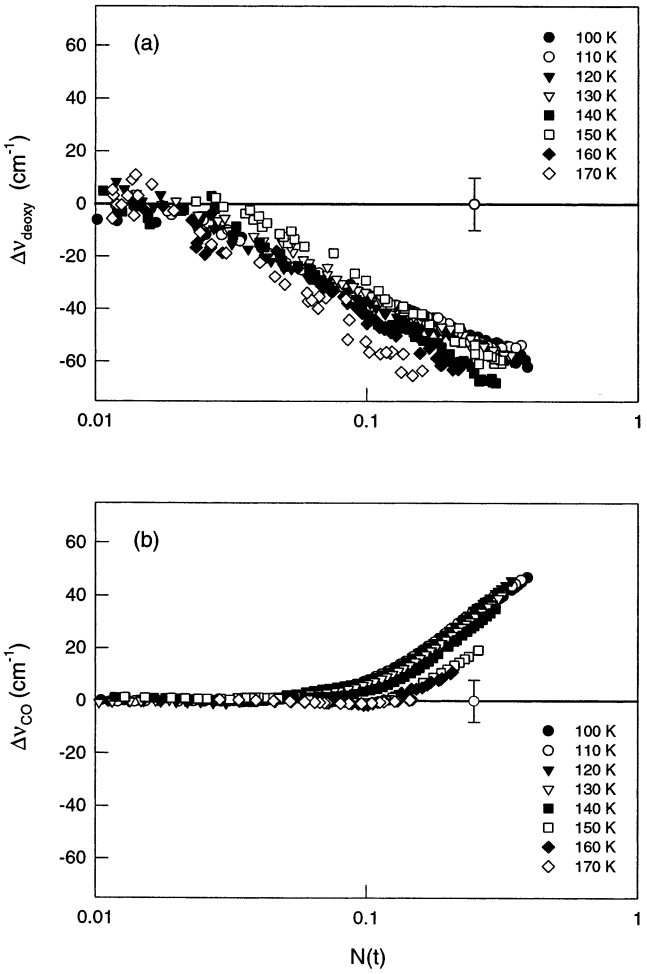

FIGURE 6: Peak frequency shift of (a) the deoxy-component and (b) the CO-component of the Soret band vs N(t) for T-state Hb in the temperature range of 100−170 K. A typical error bar (±10 cm^{-1} for $\Delta\nu_{deoxy}$; ±7.5 cm^{-1} for $\Delta\nu_{CO}$) is reported.

170 K when 1% ≤ N(t) ≤ 40%. This unambiguously means that deoxyHb in R quaternary conformation presents no KHB. The absence of KHB effect in R-state deoxyHb can be ascribed to two mechanisms: (1) because of the fast recombination kinetics and of the time resolution of the experiment, we see only ligand rebinding from CO docking sites in which the interaction of the heme group with photolyzed CO molecule is minimal, thus resulting in weakened KHB, much like as it happens in Mb at temperatures higher than 180 K (*31*); (2) interconversion between conformational substates responsible for KHB in the deoxy-component (substates likely corresponding to different orientations of the photolyzed CO molecule in the B site above the heme plane, see ref *31*) occurs in a time scale faster than our experimental resolution even at these low temperatures. However, we stress that the presence of KHB in the CO spectral component of the same sample clearly shows that interconversion between conformational substates relevant to HbCO are still hindered in the temperature range investigated.

The $\Delta\nu$ versus N(t) plots for the sample of hemoglobin in the T quaternary conformation (Figure 6) show almost opposite features. A universal behavior is observed for $\Delta\nu_{deoxy}$ at all temperatures in the range of 100−170 K. For $\Delta\nu_{CO}$, instead, a universal behavior is observed only up to 140 K; as the temperature increases, deviations from the universal curve are observed, and at 170 K practically no peak frequency shift is observed in the CO-component of the spectrum. The presence of KHB in the T-state deoxyHb clearly shows that we see ligand rebinding from CO docking sites in which the interaction of the heme group with the photolyzed CO molecule is relevant and that, within this docking site, substates interconversion is not present. The deviations from the universal behavior observed for T-state HbCO can be explained by assuming that, at T > 140 K, interconversion between conformational substates responsible for KHB in the CO-component (e.g., substates corresponding to different orientations of the liganded CO molecule) starts within the time range of our experiment.

As a final comment, we would like to discuss our data in comparison with analogous results from the pioneering work of Friedman et al. (*14*, *15*, *32*). In these works, a sol-gel technique is used to encapsulate T-state and R-state HbCO, and kinetic properties are investigated by following the CO rebinding after flash photolysis at room (from −10 up to 25 °C) temperature. However, single wavelength monitoring was used without spectral resolution. To our knowledge, our work is the first spectrally and time-resolved study of sol-gel encapsulated T- and R-state Hb. Moreover, our spectral deconvolution procedure enables us to follow both the deoxy and the CO components in the same experiment. In this way, new information on the energy landscape (KHB) and dynamics (substates interconversion) of both equilibrium (R-state HbCO and T-state deoxyHb) and intermediate (T-state HbCO and R-state deoxyHb) hemoglobin species has been obtained.

CONCLUSION

The following picture emerges from our data. For the HbCO component, KHB mainly arises from conformational substates involving the bound CO ligand (CS1), while for the deoxyHb component, KHB arises from conformational substates involving the photolyzed CO ligand (CS2).

In R-state hemoglobin, in the temperature interval of 100−170 K, enthalpy barriers between CS1 are high enough to prevent interconversion within our time window, while enthalpy barriers between CS2 are low. The opposite is true for T-state hemoglobin: in the temperature interval of 100−170 K, enthalpy barriers between CS1 are low so that interconversion occurs within our time window; on the contrary, enthalpy barriers between CS2 are high, interconversion is not present, and KHB is observed.

This highlights the dependence of the relevant conformational substates, and of their dynamic properties, on the quaternary structure of the hemoglobin molecule. In particular, the dynamics of nonequilibrium populations (i.e., R-state deoxyHb and T-state HbCO) appears substantially enhanced (as revealed by the onset of their CS interconversion) with respect to equilibrium conformations (i.e., R-state HbCO and T-state deoxyHb). This finding may be of functional relevance in that it may contribute to the instability of intermediates and therefore facilitate the quaternary structure transitions responsible for hemoglobin cooperativity. Further studies, in particular connecting the spectroscopic and dynamic properties investigated in this work to more detailed structural features, are needed to clarify the functional relevance of our results.

REFERENCES

1. Frauenfelder, H., Fenymore, P. W., and McMahon, B. H. (2002) Hydration, slaving, and protein function, *Biophys. Chem. 98*, 35−48.
2. Brunori, M. (2000) Structural dynamics of myoglobin, *Biophys. Chem. 86*, 221−230.
3. Parak, F. G., and Nienhaus, G. U. (2002) Myoglobin, a paradigm in the study of protein dynamics, *Chem. Phys. Chem. 3*, 249−254.
4. Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., and Gunsalus, I. C. (1975) Dynamics of ligand binding to myoglobin, *Biochemistry 14*, 5355−5373.
5. Frauenfelder, H., Parak, F., and Young, R. D. (1988) Conformational substates in proteins, *Annu. Rev. Biophys. Biophys. Chem. 17*, 451−479.
6. Frauenfelder, H., Sligar, S. G., and Wolynes, P. G. (1991) The energy landscapes and motions of proteins, *Science 254*, 1598−1603.
7. Steinbach, P. J., Ansari, A., Berendzen, J., Braunstein, D., Chu, K., Cowen, B. R., Ehrenstein, D., Frauenfelder, H., Johnson, J. B., Lamb, D. C., Luck, S., Mourant, J. R., Nienhaus, G. U., Ormos, P., Philipp, R., Xie, A., and Young, R. D. (1991) Ligand binding to heme proteins: the connection between dynamics and function, *Biochemistry 30*, 3988−4001.
8. Franzen, S., and Boxer, S. G. (1997) On the origin of heme absorption band shifts and associated protein structural relaxations in myoglobin following flash photolysis, *J. Biol. Chem. 272*, 9655−9660.
9. Ansari, A., Jones, C. M., Henry, E. R., Hofrichter, J., and Eaton, W. A. (1992) The role of solvent viscosity in the dynamics of protein conformational changes, *Science 256*, 1796−1798.
10. Di Iorio, E. E., Hiltpold, U. R., Filipovic, D., Winterhalter, K. H., Gratton, E., Vitrano, E., Cupane, A., Leone, M., and Cordone, L. (1991) Protein dynamics. Comparative investigation on hemeproteins with different physiological roles, *Biophys. J. 59*, 742−754.
11. Shibayama, N., and Saigo., S. (1995) Fixation of the quaternary structures of human adult haemoglobin by encapsulation in transparent porous silica gels, *J. Mol. Biol. 251*, 203−209.
12. Shibayama, N. (1999) Functional analysis of hemoglobin molecules locked in doubly liganded conformations, *J. Mol. Biol. 285*, 1383−1388.
13. Bettati, S., and Mozzarelli, A. (1997) T state hemoglobin binds oxygen noncooperatively with allosteric effects of protons, inositol hexaphosphate, and chloride, *J. Biol. Chem. 272*, 32050−32055.
14. Juszczak, L. J., and Friedman, J. M. (1999) UV resonance Raman spectra of ligand binding intermediates of sol−gel encapsulated hemoglobin, *J. Biol. Chem. 274*, 30357−30360.
15. Khan, I., Shannon, C. F., Dantsker, D., Friedman, A. J., Perez-Gonzalez-de-Apodaca, J., and Friedman, J. M. (2000) Sol-gel trapping of functional intermediates of hemoglobin: geminate and bimolecular recombination studies, *Biochemistry 39*, 16099−16109.
16. Bruno, S., Bonaccio, M., Bettati, S., Rivetti, C., Viappiani, C., Abbruzzetti, S., and Mozzarelli, A. (2001) High and low oxygen affinity conformations of T state hemoglobin, *Protein Sci. 10*, 2401−2407.
17. Campbell, B. F., Chance, M. R., and Friedman, J. M. (1987) Linkage of functional and structural heterogeneity in proteins: dynamic hole burning in carboxymyoglobin, *Science 238*, 373−376.
18. Chavez, M. D., Courtney, S. H., Chance, M. R., Kiula, D., Nocek, H., Hoffman, B. M., Friedman, J. M., and Ondrias, M. R., (1990) Structural and functional significance of inhomogeneous line broadening of band III in hemoglobin and Fe−Mn hybrid hemoglobins, *Biochemistry 29*, 4844−4852.
19. Srajer, V., and Champion, P. M. (1991) Investigation of optical line shapes and kinetic hole burning in myoglobin, *Biochemistry 30*, 7930−7402.
20. Huang, J., Ridsdale, A., Wang, J., and Friedman, J. M. (1997) Kinetic hole burning, hole filling, and conformational relaxation in heme proteins: direct evidence for the functional significance of a hierarchy of dynamical processes, *Biochemistry 36*, 14353−14365.
21. Cupane, A., Leone, M., Vitrano, E., and Cordone, L. (1995) Low-temperature optical absorption spectroscopy: an approach to the study of stereodynamic properties of hemeproteins, *Eur. Biophys. J. 23*, 385−398.
22. Cordone, L., Cupane, A., San Biagio, P. L., and Vitrano, E. (1979) Effect of some monohydric alcohols on the oxygen affinity of hemoglobin: relevance of solvent dielectric constant and hydrophobicity, *Biopolymers 18*, 1975−1988.
23. Ellerby, L. M., Nishida, C. R., Nishida, F., Yamanaka, S. A., Dunn, B., Valentine, J. S., and Zink, J. I. (1992) Encapsulation of proteins in transparent porous silicate glasses prepared by the sol-gel method, *Science 255*, 1113−1115.
24. Chan, C. K., and Page, J. B. (1983) Temperature effects in the time correlator theory of resonance Raman scattering, *J. Chem. Phys. 79*, 5234−5250.
25. Srajer, V., Schomacker, K. T., and Champion, P. M. (1986) Spectral broadening in biomolecules, *Phys. Rev. Lett. 57*, 1267−1270.
26. Cupane, A., Vitrano, E., Ormos, P., and Nienhaus, G. U. (1996) Heme geometry in the 10 K photoproduct from sperm whale carbonmonoxy-myoglobin, *Biophys. Chem. 60*, 111−117.
27. Rausei, M. Effect of quaternary structure on the dynamic properties of hemoglobin, Ph.D. Thesis, University of Palermo, Palermo, Italy, 1999.
28. Ormos, P., Szaraz, S., Cupane, A., and Nienhaus, G. U. (1998) Structural factors controlling ligand binding to myoglobin: a kinetic hole-burning study, *Proc. Natl. Acad. Sci. U.S.A. 95*, 6762−6767.
29. Agmon, N. (1988) Reactive line-shape narrowing in low-temperature inhomogeneous geminate recombination of CO to myoglobin, *Biochemistry 27*, 3507−3511.
30. Ansari, A. Conformational relaxation and kinetic hole-burning in sperm whale myoglobin, Ph.D. Dissertation, University of Illinois, Urbana-Champaign, 1989.
31. Nienhaus, K., Lamb, D. C., Deng, P., and Nienhaus, G. U. (2002) The Effect of ligand dynamics on heme electronic transition band III in myoglobin, *Biophys. J. 82*, 1059−1067.
32. Samuni, U., Dantsker, D., Khan, I., Friedman, A. J., Peterson, E., and Friedman, J. M. (2002) Spectroscopically and kinetically distinct conformational populations of sol-gel-encapsulated carbonmonoxy myoglobin. A comparison with hemoglobin, *J. Biol. Chem. 277*, 25783−25790.

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