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Biophysical Chemistry 114 (2005) 27–33

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Spectroscopic markers of the T↔R quaternary transition in human hemoglobin

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Received 7 October 2004; received in revised form 4 November 2004; accepted 4 November 2004

Abstract

In this work, we use a sol–gel protocol to trap and compare the R and T quaternary states of both the deoxygenated (deoxyHb) and carbonmonoxide (HbCO) derivatives of human hemoglobin. The near infrared optical absorption band III and the infrared CO stretching band are used to detect the effect of quaternary structure on the spectral properties of deoxyHb and HbCO; comparison with myoglobin allows for an assessment of tertiary and quaternary contributions to the measured band shifts. The R↔T transition is shown to cause a blue shift of the band III by ~35 cm^{-1} for deoxyHb and a red shift of the CO stretching band by only ~0.3 cm^{-1} for HbCO. This clearly shows that quaternary structure changes are transmitted to the heme pocket and that effects on deoxyHb are much larger than on HbCO, at least as far as the band energies are concerned. Experiments performed in the ample temperature interval of 300–10K show that the above quaternary structure effects are "static" and do not influence the dynamic properties of the heme pocket, at least as probed by the temperature dependence of band III and of the CO stretching band. The availability of quaternary structure sensitive spectroscopic markers and the quantitative measurement of the quaternary structure contribution to band shifts will be of considerable help in the analysis of flash-photolysis experiments on hemoglobin. Moreover, it will enable one to characterize the dynamic properties of functionally relevant hemoglobin intermediates and to study the kinetics of both the T→R and R→T quaternary transitions through time-resolved spectroscopy.

Keywords: FTIR spectroscopy; Band III; CO stretching band; Sol–gel encapsulation; Low temperature spectroscopy

1. Introduction

Quaternary conformational changes are of utmost importance to protein function. The best known example is probably the T↔R transition of hemoglobin, which is responsible for the cooperative ligand binding of this protein [1]. To monitor the conformational changes involved in hemoglobin function, it is mandatory to have experimentally accessible markers that not only depend on the structural details but also enable one to distinguish between spectral contributions arising from different levels of protein structure (i.e., tertiary vs. quaternary) and from its ligation state. Decoupling the protein quaternary conformation from ligation state is of particular relevance in the case of hemoglobin since it may help identifying and characterizing intermediate species (e.g., T-state liganded or R-state unliganded hemoglobin) that are relevant to understand the functional behavior of this protein.

The search for quaternary structure sensitive spectroscopic markers has a long story. In the classical studies of Perutz et al. [2–5], ferric hemoglobin and/or mutant or chemically modified ferrous hemoglobins in the presence and absence of the allosteric effector inositol-hexaphosphate (IHP) were used to find quaternary structure sensitive spectroscopic markers. Iron–cobalt hybrid hemoglobins have also been investigated to study the effects of quaternary structure on various spectroscopic properties [6,7].

More recently, sol–gel encapsulation protocols [8–10] have been used to dramatically slow down the kinetics of

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doi:10.1016/j.bpc.2004.11.005

quaternary conformational changes; in fact, at ~4 °C and in the presence of viscous solvents, the characteristic time of quaternary transition has been shown to be of the order of several days [9–11]; on the other hand, the encapsulated protein exhibits almost unaltered spectral and functional properties [8,10]. Since the ligation state of sol–gel encapsulated hemoglobin can be changed in much shorter times (milliseconds to seconds), this effectively decouples the protein quaternary conformation from its ligation state and allows to investigate the quaternary transition at constant ligation. In particular, Shibayama and Saigo [11] have used difference spectra in the Soret region to monitor the R→T transition; however, spectral shifts were not quantitatively measured, and contributions from tertiary and/or quaternary structure were not singled out. The iron-proximal histidine stretching frequency ($\nu_{\text{Fe-His}}$) measured in resonance Raman experiments has been used by Friedman and coworkers [9,12,13] to monitor the effect of quaternary structure on sol–gel encapsulated hemoglobin and on the photoproducts obtained after flash-photolysis of the carbon-monoxy derivatives. $\nu_{\text{Fe-His}}$ is indeed a fine probe of the proximal heme pocket conformation and enables one to measure quantitatively the spectral shifts; however, it is highly sensitive to effects of both tertiary and quaternary structures and the separation of the above effects is often difficult.

On the other hand, the infrared stretching band of the bound CO molecule and the near infrared absorption band III are typical spectroscopic markers of hemoglobin (Hb) and myoglobin (Mb) conformation and could therefore be used as quaternary structure sensitive spectroscopic markers. The first band is located at about 1950 cm^{-1} and is a fine probe of the distal heme pocket conformation; it has been used, e.g., to characterize the so-called taxonomic substates in MbCO [14] and to probe the orientation of the bound CO molecule [15–17] and/or the electrostatic environment of the heme [18]. For HbCO, the CO stretching band of the native protein and of several mutants has been studied [19,20]; for native human HbCO, the thermal behavior in the temperature interval of 300–10 K has also been investigated [21]. The effects of protein quaternary conformation on the peak position and shape of this band have been reported only for the mutant hemoglobin Kansas and for carp hemoglobin, with or without IHP [22], and for T-state human hemoglobin crystals at low CO saturation [23].

Band III is a porphyrin-to-iron charge transfer band [24] near 760 nm (~13,160 cm^{-1}) and is observed only in the deoxygenated derivatives. As early as 1974, Iizuka et al. [25] noticed that, in the 4.2 K photoproduct of MbCO and HbCO, band III is substantially red shifted with respect to the equilibrium deoxy-proteins values; these authors suggested that band III is sensitive to protein structure and called it the "conformation band". Band III has been used to characterize the structure and relaxation of the photoproduct generated by photolysis of MbCO both at cryogenic and room temperature [26]. The various contributions to the spectral shift arising from kinetic hole burning, protein structural relaxation and ligand migration have been investigated by using time resolved optical absorption spectroscopy [27] and temperature derivative spectroscopy [28]; a recent paper by Nienhaus et al. [29] assigns ~60 cm^{-1} red shift to kinetic hole burning and ~60 cm^{-1} to protein relaxation and/or ligand migration. As far as Hb is concerned, the situation is further complicated by the possible presence of quaternary relaxation. In 1987, Sassaroli and Rousseau [30] showed that the position of band III after the photolysis of HbCO at room temperature is red shifted with respect to the deoxy equilibrium value by ~6 nm at 10 ns and is still red shifted by ~2 nm at 50 μs; this is different from Mb, in which, at room temperature, band III has almost completely relaxed to the deoxy value already at 10 ns from photolysis [31]. Further studies [32] do not allow to estimate quantitatively the contribution of quaternary relaxation to the observed shifts. Indeed, results of flash-photolysis experiments are, in general, difficult to interpret in view of the simultaneous presence of structural relaxation(s) and ligand rebinding [33,34].

In this work, we extend the sol–gel approach to prepare samples in which Hb is encapsulated at sufficiently high concentration to study the effect of quaternary structure on both the CO stretching band and band III. Our approach enables one to study quaternary structure effects both on ligated and deoxy hemoglobin and to measure quantitatively the spectral shifts. Experiments are performed in the ample temperature interval of 300–10 K to get information also on the dynamic properties of encapsulated proteins.

2. Materials and methods

2.1. Samples

Human Hb was prepared and stored in the oxygenated form following a standard procedure previously described [35]. HbO_2 (~30 wt.%) was suitably diluted to obtain an oxyhemoglobin solution ~5 mM (in heme) in 54% v/v glycerol and 0.2 M K-phosphate buffer at pH 7 (solution 1). A mixture of 60% tetramethylorthosilicate, 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 solution 1. The gel is formed in about 1 min at ~7 °C; after gelification, the gel is covered with a protective solution (40% v/v glycerol, 0.2 M K-phosphate buffer, pH 7), sealed and left to age for 10 h at 7 °C. This encapsulation protocol ensures that the protein is indeed encapsulated within the silica matrix and not just adsorbed on it. To increase solvent viscosity, the protective solution is then substituted with an otherwise identical solution containing 70% v/v glycerol, and the gel is left to equilibrate overnight at 7 °C. The effective sample equilibration with the protective solution was verified by comparing the intensities of glycerol and

water IR absorption bands. The samples were maintained at 7 °C during all the preparation steps; this fact, together with the rather high glycerol concentration used, ensures that the hemoglobin encapsulated in our samples retains its original quaternary conformation. In fact, it has been shown that, at low temperatures and in the presence of viscous solvents, the kinetics of quaternary conformational changes in sol–gel encapsulated hemoglobin is slowed down to characteristic times of the order of weeks or months [9,10,12].

From the above general procedure, four different kinds of Hb gels were prepared for this study:

Sample 1: T-state deoxyHb. Solution 1, after equilibration with N_2 for ~30 min and the anaerobic addition of ~50 mM Na-dithionite, to have a fully reduced sample, was mixed with the sonicated sol and layered on the inner face of a metacrylate cuvette. Protective solutions (40% and 70% v/v glycerol) were saturated with N_2 and contained ~50 mM Na-dithionite.

Sample 2: R-state deoxyHb. The standard procedure for gel formation was followed, and the HbO_2 gel was layered on the inner face of a metacrylate cuvette. Immediately before the experiment, the HbO_2 gel was deoxygenated by N_2 bubbling and anaerobic addition of ~50 mM Na-dithionite; full deoxygenation occurred in about 2 h at 7 °C.

Sample 3: T-state HbCO. A deoxyHb gel was prepared as for sample 1 and layered on a CaF_2 window having a 0.1-mm spacer. Immediately before the experiment, the gel was equilibrated with CO by gentle bubbling; full sample equilibration occurred in about 1 h at 7 °C.

Sample 4: R-state HbCO. This sample was prepared exactly as sample 1, with the difference that CO was used as the saturating gas instead of N_2 and that the gel was layered on a CaF_2 window having a 0.1-mm spacer.

The amount of methemoglobin in each sample was checked at the end of each experiment and found to be always less than 5%. Careful filling of the cuvettes avoided problems related to bubble trapping; the absence of bubbles was checked at the end of each experiment.

2.2. Spectroscopy

FTIR spectra of HbCO samples were measured with a Jasco FTIR-410 spectrometer. After gentle drying, the CaF_2 window with the gel was sealed and mounted on the sample holder of an Oxford cryostat for measurements in the temperature interval 300–10 K. The temperature was controlled and measured by an Oxford ITC-503 temperature controller.

Single-beam spectra in the 1000–4000 cm^{-1} wave numbers range were measured with 300 scans at 1 cm^{-1} resolution. The single-beam "blank" spectra of a deoxyHb gel were also measured at the same temperatures in a separate experiment. The absorption spectra of blank and samples were calculated with respect to the single beam of the empty cell; at each temperature, the blank spectrum was subtracted from the sample spectrum after suitable normalization, to eliminate the contributions of nearby protein and solvent bands.

Optical absorption spectra in the 720–800 nm wavelength interval were measured with a Jasco V-570 spectrophotometer using the already described apparatus for optical absorption measurements in the temperature interval 300–10 K [36]. To take into account the background, a cubic baseline was subtracted from the measured spectra using the Peakfit package.

As usual [37,38], the moments of the bands were calculated as:

$$M_0 = \int A(\nu)\mathrm{d}\nu$$

$$M_1 = \int \nu A(\nu)\mathrm{d}\nu / M_0$$

$$M_2 = \int \nu^2 A(\nu)\mathrm{d}\nu / M_0 - {M_1}^2$$

3. Results and discussion

The room temperature FTIR spectra of HbCO encapsulated in T and R conformations (samples 3 and 4) are shown in Fig. 1. For both samples, a single band at ~1953 cm^{-1} accounts for more than 90% of the spectrum, in agreement with previous results on R-state HbCO in solution [21]. The main band can be attributed to substate A_1, while the smaller band at ~1970 cm^{-1} can be attributed to substate A_0. For a more in-depth discussion about the nature of the taxonomic substates A_0 and A_1 in MbCO and HbCO, we refer the reader to Refs. [39,20], respectively. The R→T transition

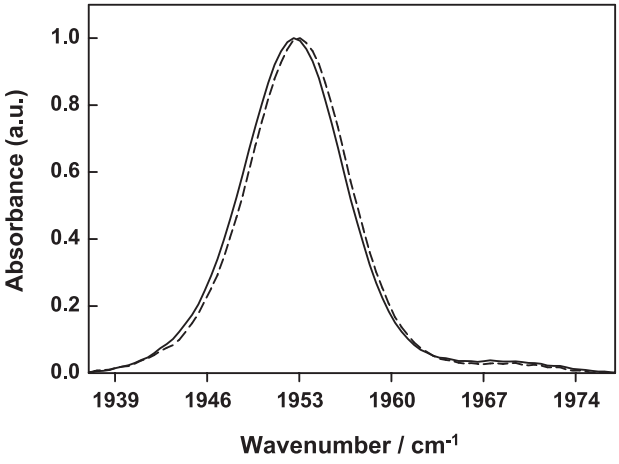

Fig. 1. Absorption spectra of HbCO in the CO stretching region at T=300 K. Continuous line: T-state HbCO (Sample 3); dashed line: R-state HbCO (Sample 4).

does not alter appreciably the A_0 band nor the A_0/A_1 intensity ratio (at least within the resolution of our experiment); it causes a small frequency shift in the A_1 band, with T-state HbCO being red shifted by ~0.3 cm^{-1} with respect to R-state HbCO, while the overall band width remains unaltered. In view of the smallness of the effect, this experiment was repeated several times; data in Fig. 1 are the result of the average of seven coupled experiments on R-state and T-state HbCO. From this average, we estimate a redshift of 0.3 ± 0.2 cm^{-1}.

From the FTIR spectra measured at various temperatures, the zeroth (M_0), first (M_1) and second (M_2) moments of the A_1 band were calculated following the procedure reported in Ref. [21], and their temperature dependence is reported in Fig. 2. The only sizeable effect is a temperature-independent M_1 red shift of T-state HbCO with respect to R-state HbCO, while M_0 and M_2 are largely unaffected. The lack of any sizeable effect on the M_2 thermal behavior suggests that the local active site dynamic properties of T-state and R-state HbCO (at least as probed by the bound CO stretching band) are very similar and that the effect of quaternary structure on the heme pocket of HbCO responsible for the M_1 shift reported in Fig. 1 and in panel (b) of Fig. 2 is static and not dynamic.

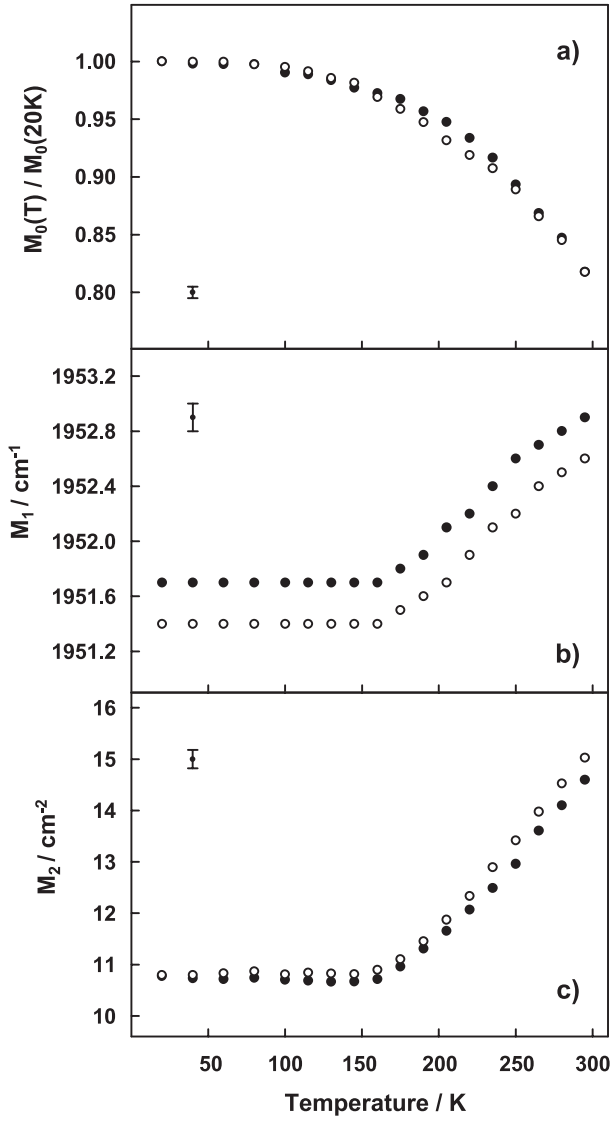

Fig. 2. Temperature dependence of the zeroth (M_0, panel a), first (M_1, panel b) and second (M_2, panel c) moments of the CO stretching band (A_1 band). (●): R-state HbCO (Sample 4); (○): T-state HbCO (Sample 3).

The quaternary structure dependence of the C–O stretching frequency has been previously studied using the carbonmonoxy derivatives of the mutant hemoglobin Kansas and of carp hemoglobin in the presence and absence of IHP [22]. For hemoglobin Kansas, the R→T transition induced by the addition of IHP caused a ~0.8 cm^{-1} redshift; on the contrary, a ~0.7 cm^{-1} blueshift was observed for carp hemoglobin. More recently, polarized IR measurements on T-state hemoglobin crystals at low CO saturation [23] have shown that, in the T-state crystal, the CO bands are broader and lower in frequency by about 0.8 cm^{-1} than in the R-state HbCO solution; moreover, the A_0/A_1 intensity ratio is substantially increased. This last effect was attributed to a partial disruption of the β-chains distal heme pocket upon CO binding to β hemes in the T conformation. Comparison with the present data indicates that sol–gel encapsulation introduces less severe constraints on the distal heme pocket conformation with respect to the crystal. In fact, sol–gel encapsulated T-state HbCO (sample 3) has the same band width (i.e., conformational heterogeneity) and A_0/A_1 population ratio than that of the sol–gel encapsulated R-state HbCO and R-state HbCO in the solution. On the other hand, data in Fig. 1 show that, for sol–gel encapsulated human HbCO, a smaller effect (~0.3 cm^{-1} redshift) is observed; in view of the species variability previously observed, this could be typical of human hemoglobin. However, in view of the rather long CO equilibration times used, the presence of tertiary relaxation in T-state HbCO (sample 3) cannot be excluded. In any case, the low temperature used for CO equilibration and the high solvent viscosity exclude the possibility of significant quaternary structure relaxation [9]. In agreement with previous interpretations [20,40,41], the small shift in band position can be attributed to slightly different local electrostatic environments of CO bound to T-state and R-state hemoglobin. Data in Fig. 1, however, indicate that this effect is small.

Fig. 3a reports band III of T-state and R-state deoxyHb in the gel (samples 1 and 2), together with analogous data relative to deoxyHb in 70% glycerol/water solution. Band III measured in the R-state deoxyHb gel is red shifted by ~35 cm^{-1} (~2 nm) with respect to the T-state deoxyHb gel. Moreover, the T-state deoxyHb gel spectrum is almost indistinguishable from solution one at all temperatures investigated (see also Fig. 4), thus indicating that the gel encapsulation procedure *per se* does not introduce any relevant spectral shift nor alteration of the dynamic properties. It also suggests that, in agreement with previous works [8,10], the structural properties of encapsulated hemoglobin are unaffected. In several days, band III gradually relaxes

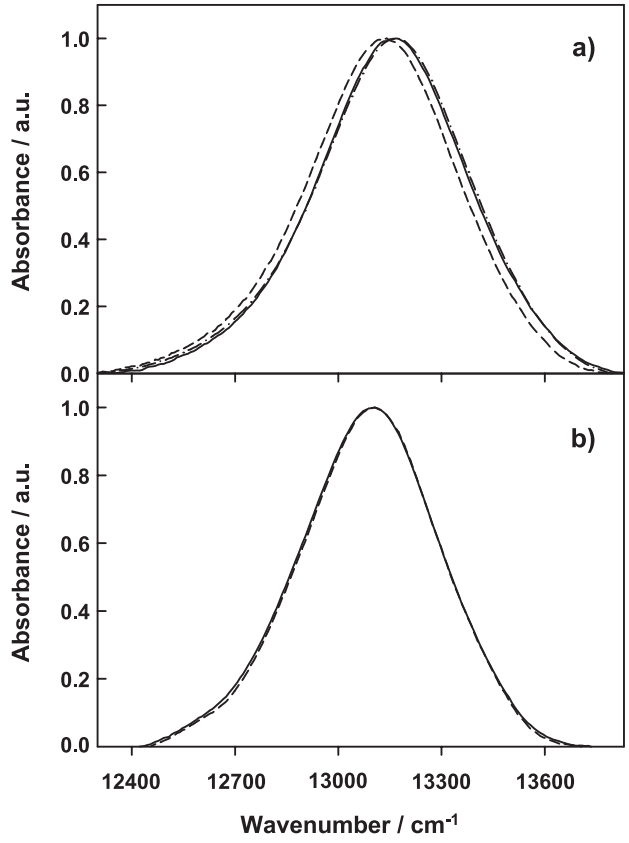

Fig. 3. (a) Band III of the T-state deoxyHb in gel (continuous line, Sample 1), of R-state deoxyHb in gel (dashed line, Sample 2) and of T-state deoxyHb in a 70% v/v glycerol/water solution (dashed-dotted line). (b) Same as in Panel (a), for deoxyMb. Encapsulation protocols for Mb were analogous as for Hb. T=298 K.

from the R-state spectrum towards the T-state one; the kinetics of spectral relaxation will be reported in a further publication. Fig. 3b reports analogous spectra relative to Mb and clearly shows that, for this protein, no difference is observed between the anaerobically and aerobically prepared sol–gel samples. This indicates that the ~35 cm^{-1} shift observed for Hb cannot be attributed to encapsulation-induced differences in tertiary structure.

The temperature dependence of M_0, M_1 and M_2 relative to band III is reported in Fig. 4. In analogy with the data relative to the CO stretching band (see Fig. 2), the only sizeable effect, is a temperature-independent M_1 shift. In this case, however, R-state deoxyHb is red shifted with respect to T-state deoxyHb. Data in Fig. 4 indicate that, also for deoxyHb, the effect of quaternary structure on the heme pocket (at least as monitored by the first moment of band III) is static and not dynamic.

Data in Fig. 3 should be compared with resonance Raman spectroscopy data on sol–gel encapsulated deoxyHb [9,12]. In these works, the frequency of the iron-proximal histidine stretching mode, $\nu_{Fe\text{-}His}$, of both T-state and R-state deoxyHb was measured and compared with deoxyMb. For T-state deoxyHb in gel, $\nu_{Fe\text{-}His}$ is 214 cm^{-1}, identical to the value measured for deoxyHb in solution; for R-state deoxyHb in gel, $\nu_{Fe\text{-}His}$ is 221.8–223 cm^{-1}, to be compared with the value of 230 cm^{-1} observed in the 8-ns photoproduct of HbCO in solution. Moreover, in time-resolved resonance Raman experiments performed after the deoxygenation of an encapsulated oxyHb sample [9], two relaxations were observed: the first one, occurring between 1 and 100 min after deoxygenation, was attributed to the relaxation of the protein tertiary structure, while the second one, occurring at much longer times (~1 day, at 22 °C), was assigned to the R→T quaternary structure transition. The value of 221.8–223 cm^{-1} measured for R-state deoxyHb in the "static" experiment of Ref. [12] corresponds to the plateau value observed between the two relaxations. In view of the above results and of the rather long deoxygenation time needed in our experiment (~2 h), tertiary relaxation in

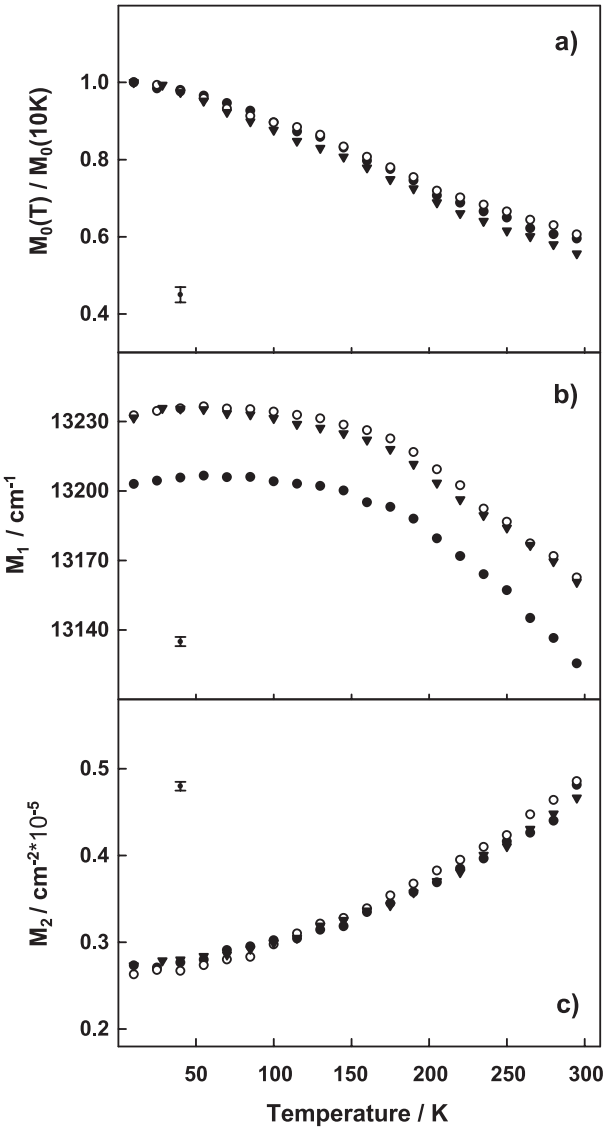

Fig. 4. Temperature dependence of the zeroth (M_0, panel a), first (M_1, panel b) and second (M_2, panel c) moments of band III. (●): R-state deoxyHb; (○): T-state deoxyHb; values relative to T-state deoxyHb in a 70% v/v glycerol/water solution (▼) are also reported, for comparison.

sample 3 is certainly faster than our time resolution, and therefore, the ~35 cm^{-1} band III shift has to be attributed to the different quaternary structure; this interpretation is strongly supported by the data relative to Mb, in which no spectral shift is observed.

Further insights can be obtained by comparison with the time-resolved spectral data on band III by Sassaroli and Rousseau [30]. These authors investigated the spectral relaxation of band III at room temperature in photolyzed HbCO. At 10 ns after photolysis, band III is red shifted with respect to the equilibrium deoxy value by about 6 nm (~105 cm^{-1}); between 10 and 100 ns, a marked blueshift is observed, followed by a plateau region extending up to 10 μs, still showing a ~2 nm (~35 cm^{-1}) redshift with respect to the equilibrium deoxy value. It is remarkable that 35 cm^{-1} is just the spectral redshift between the R-state and T-state deoxyHb measured in our experiment; in view of the data reported in our work, the residual redshift observed by Sassaroli and Rousseau can therefore be safely attributed to the effect of quaternary structure.

Putting together the results of Refs. [9,12,30] with those of our experiment, the following picture emerges. After deoxygenation of liganded hemoglobin (either by flash photolysis or by reduction with dithionite), both tertiary and quaternary relaxations contribute to the spectral shifts observed in band III. As shown by time-resolved optical absorption [30] and resonance Raman data [42,43], at room temperature and for hemoglobin in solution, tertiary relaxation occurs in the time range of 10–100 ns, while the R→T quaternary relaxation occurs at about 20 μs. For band III, tertiary relaxation contributes ≥100 cm^{-1} to the overall spectral shift, while quaternary relaxation contributes ~35 cm^{-1}. Encapsulation within a silica matrix slows down both relaxations. Resonance Raman data on $\nu_{Fe\text{-}His}$ show that, with the encapsulation protocol of Das et al. [9] and at 22 °C, tertiary and quaternary relaxations occur at about 10 and 1000 min, respectively. Our data show that, with our encapsulation protocol and at 25 °C, tertiary and quaternary relaxations are much faster and much slower than ~60 min, respectively. Therefore, there is qualitative agreement between the two sets of data.

4. Conclusions

We think that the reported data are relevant in several respects:

(a) They clearly show that quaternary structure changes are transmitted to the heme pocket; effects on deoxygenated Hb, however, are much larger than on HbCO ($\Delta\nu/\nu$ values for band III are ~20 times larger than for the CO stretching band). It must be stressed that "much larger" refers only to effects on the energies of the bands investigated, as measured by fractional peak frequency shifts ($\Delta\nu/\nu$ values); no direct information on the extent of, e.g., structural effects can be obtained from our data alone.

(b) The effect of the T ↔ R quaternary transition is static and likely involves alterations in the electrostatic environment of the heme pocket; dynamic properties, as probed by the temperature dependence of the bands moments, are largely unaffected.

(c) This is the first quantitative measurement of the quaternary structure contribution to the band III shift and may be of considerable help in the analysis of flash-photolysis experiments on Hb [30,32,34].

(d) the availability of quaternary structure sensitive spectroscopic markers, together with sol–gel encapsulation protocols, will enable one to characterize the dynamic properties of functionally relevant Hb intermediates otherwise barely accessible [13,44] and to study the kinetics of both the T→R and R→T quaternary transitions through time-resolved spectral relaxation measurements.

References

[1] M.F. Perutz, A.J. Wilkinson, M. Paoli, G.G. Dodson, The stereochemical mechanism of the cooperative effects in hemoglobin revisited, Annu. Rev. Biophys. Biomol. Struct. 27 (1998) 1–34.
[2] M.F. Perutz, J.E. Ladner, S.R. Simon, C. Ho, Influence of globin structure on the state of the heme: I. Human deoxyhemoglobin, Biochemistry 13 (1974) 2163–2173.
[3] M.F. Perutz, A.R. Fersht, S.R. Simon, G.C. Roberts, Influence of globin structure on the state of the heme: II. Allosteric transitions in methemoglobin, Biochemistry 13 (1974) 2174–2186.
[4] M.F. Perutz, E.J. Heidner, J.E. Ladner, J.G. Beetlestone, C. Ho, E.F. Slade, Influence of globin structure on the state of the heme: III. Changes in heme spectra accompanying allosteric transitions in methemoglobin and their implications for heme–heme interactions, Biochemistry 13 (1974) 2187–2200.
[5] M.F. Perutz, J.V. Kilmartin, K. Nagai, A. Szabo, Influence of globin structure on the state of the heme, Biochemistry 15 (1976) 378–387.
[6] M.R. Ondrias, D.L. Rousseau, T. Kitagawa, M. Ikeda-Saito, T. Inubushi, T. Yonetani, Quaternary structure changes in iron–cobalt hybrid hemoglobins detected by resonance Raman scattering, J. Biol. Chem. 257 (1982) 8766–8770.
[7] L.P. Murray, J. Hofrichter, E.R. Henry, M. Ikeda-Saito, K. Kitagishi, T. Yonetani, W.A. Eaton, The effect of quaternary structure on the kinetics of conformational changes and nanosecond geminate rebinding of carbon monoxide to hemoglobin, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 2151–2155.
[8] N. Shibayama, S. Saigo, Fixation of the quaternary structures of human adult haemoglobin by encapsulation in transparent porous silica gels, J. Mol. Biol. 251 (1995) 203–209.
[9] T.K. Das, I. Khan, D.L. Rousseau, J.M. Friedman, Temperature dependent quaternary state relaxation in sol–gel encapsulated hemoglobin, Biospectroscopy 5 (1999) S64–S70.
[10] S. Bettati, A. Mozzarelli, T state hemoglobin binds oxygen non-cooperatively with allosteric effects of protons, inositol hexaphosphate, and chloride, J. Biol. Chem. 272 (1997) 32050–32055.
[11] N. Shibayama, S. Saigo, Kinetics of the allosteric transition in hemoglobin within silicate sol–gels, J. Am. Chem. Soc. 121 (1999) 444–445.
[12] U. Samuni, D. Dantsker, I. Khan, A.J. Friedman, E. Peterson, J.M. Friedman, Spectroscopically and kinetically distinct conforma-

tional populations of sol–gel encapsulated carbonmonoxy myoglobin: a comparison with hemoglobin, J. Biol. Chem. 277 (2002) 25783–25790.
[13] I. Khan, C.F. Shannon, D. Dantsker, A.J. Friedman, J. Perez-Gonzalez-de-Apodaca, J.M. Friedman, Sol–gel trapping of functional intermediates of hemoglobin: geminate and bimolecular recombination studies, Biochemistry 39 (2000) 16099–16109.
[14] J.B. Johnson, D.C. Lamb, H. Frauenfelder, J.D. Muller, B. McMahon, G.U. Nienhaus, R.D. Young, Ligand binding to heme proteins: 6. Interconversion of taxonomic substates in carbonmonoxymyoglobin, Biophys. J. 71 (1996) 1563–1573.
[15] P. Ormos, D. Braunstein, H. Frauenfelder, M.K. Hong, S.L. Lin, T.B. Sauke, R.D. Young, Orientation of carbon monoxide and structure–function relationship in carbonmonoxymyoglobin, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 8492–8496.
[16] M. Lim, T.A. Jackson, P.A. Anfinrud, Orientational distribution of CO before and after photolysis of MbCO and HbCO: a determination using time-resolved polarized Mid-IR spectroscopy, J. Am. Chem. Soc. 126 (2004) 7946–7957.
[17] M. Lim, T.A. Jackson, P.A. Anfinrud, Binding of CO to myoglobin from a heme pocket docking site to form nearly linear Fe–C–O, Science 269 (1995) 962–966.
[18] J.T. Sage, D. Morikis, P.M. Champion, Spectroscopic studies of myoglobin at low pH: heme structure and ligation, Biochemistry 30 (1991) 1227–1237.
[19] W.T. Potter, J.H. Hazzard, M.G. Choc, M.P. Tucker, W.S. Caughey, Infrared spectra of carbonyl hemoglobins: characterization of dynamic heme pocket conformers, Biochemistry 29 (1990) 6283–6295.
[20] M. Karavitis, C. Fronticelli, W.S. Brinigar, G.B. Vasquez, V. Militello, M. Leone, A. Cupane, Properties of human hemoglobins with increased polarity in the alpha- or beta-heme pocket - Carbonmonoxy derivatives, J. Biol. Chem. 273 (1998) 23740–23749.
[21] A. Cupane, M. Leone, V. Militello, Conformational substates and dynamic properties of carbonmonoxy hemoglobin, Biophys. Chem. 104 (2003) 335–344.
[22] D.L. Rousseau, S.L. Tan, M.L. Ondrias, S. Ogawa, R.W. Noble, Absence of cooperative energy at the heme in liganded hemoglobins, Biochemistry 23 (1984) 2857–2865.
[23] H. Khachfe, M. Mylrajan, J.T. Sage, Infrared investigation of T-state hemoglobin, Cell. Mol. Biol. 44 (1998) 39–52.
[24] W.A. Eaton, J. Hofrichter, Polarized absorption and linear dichroism spectroscopy of hemoglobin, Methods Enzymol. 76 (1981) 175–261.
[25] T. Iizuka, H. Yamamoto, M. Kotani, T. Yonetani, Low temperature photodissociation of heme proteins: carbon monoxide complex of myoglobin and hemoglobin, Biochim. Biophys. Acta 371 (1974) 2667–2679.
[26] G.U. Nienhaus, J.R. Mourant, K. Chu, H. Frauenfelder, Ligand binding to heme proteins. The effect of light on ligand binding to myoglobin, Biochemistry 33 (1994) 13413–13430.
[27] M. Levantino, A. Cupane, L. Zimanyi, P. Ormos, Different relaxations in myoglobin after photolysis, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 14402–14407.
[28] P. Ormos, S. Szaraz, A. Cupane, G.U. Nienhaus, Structural factors controlling ligand binding to myoglobin, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 6762–6767.
[29] K. Nienhaus, D.C. Lamb, P. Deng, G.U. Nienhaus, The effects of ligand dynamics on heme electronic transition band III in myoglobin, Biophys. J. 82 (2002) 1059–1067.
[30] M. Sassaroli, D.L. Rousseau, Time dependence of near-infrared spectra of photodissociated hemoglobin and myoglobin, Biochemistry 26 (1987) 3092–3098.
[31] M. Lim, T.A. Jackson, P.A. Anfinrud, Non exponential protein relaxation: dynamics of conformational change in myoglobin, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5801–5804.
[32] M.D. Chavez, S.H. Courtney, M.R. Chance, D. Kiula, J. Nocek, B.M. Hoffman, J.M. Friedman, M.R. Ondrias, Structural and functional significance of inhomogeneous line broadening of band III in hemoglobin and Fe–Mn hybrid hemoglobins, Biochemistry 29 (1990) 4844–4852.
[33] J. Hofrichter, E.R. Henry, A. Szabo, L.P. Murray, A. Ansari, C.M. Jones, M. Coletta, G. Falcioni, M. Brunori, W.A. Eaton, Dynamics of the quaternary conformational change in trout hemoglobin, Biochemistry 30 (1991) 6583–6598.
[34] C.M. Jones, A. Ansari, E.R. Henry, G.W. Christoph, J. Hofrichter, W.A. Eaton, Speed of intersubunit communication in proteins, Biochemistry 31 (1992) 6692–6702.
[35] L. Cordone, A. Cupane, P.L. San Biagio, E. Vitrano, Effect of some monohydric alcohols on the oxygen affinity of hemoglobin: relevance of solvent dielectric constant and hydrophobicity, Biopolymers 18 (1979) 1975–1988.
[36] L. Cordone, A. Cupane, M. Leone, E. Vitrano, Optical absorption spectra of deoxy- and oxyhemoglobin in the temperature range 300–20 K. Relation with protein dynamics, Biophys. Chem. 24 (1986) 259–275.
[37] L. Cordone, A. Cupane, M. Leone, E. Vitrano, Thermal behavior of the 760 nm band in photodissociated sperm whale carbonmonoxymyoglobin at cryogenic temperature: dependence on external medium, Biopolymers 29 (1990) 639–643.
[38] S.S. Stavrov, Optical absorption band III of deoxyheme proteins as a probe of their structure and dynamics, Chem. Phys. 271 (2001) 145–154.
[39] J. Vojtechovsky, K. Chu, J. Berendzen, R.M. Sweet, I. Schlichting, Crystal structures of myoglobin-ligand complexes at near-atomic resolution, Biophys. J. 77 (1999) 2153–2174.
[40] B. Kushkuley, S.S. Stavrov, Theoretical study of the distal-side steric and electrostatic effects on the vibrational characteristics of the FeCO unit of the carbonylheme proteins and their models, Biophys. J. 70 (1996) 1214–1229.
[41] G.B. Ray, X.Y. Li, J.A. Ibers, J.L. Sessler, T.G. Spiro, How far can proteins bend the Fe–CO unit. Distal polar and steric effects in heme-proteins and models, J. Am. Chem. Soc. 116 (1994) 162–176.
[42] J.W. Scott, J.M. Friedman, Tertiary-structure relaxation in hemoglobin: a transient Raman study, J. Am. Chem. Soc. 106 (1984) 5677–5687.
[43] V. Jayaraman, K.R. Rodgers, I. Mukerji, T.G. Spiro, Hemoglobin allostery: resonance Raman spectroscopy of kinetic intermediates, Science 269 (1995) 1843–1848.
[44] M. Levantino, A. Cupane, L. Zimanyi, Quaternary structure dependence of kinetic hole burning and conformational substates interconversion in hemoglobin, Biochemistry 42 (2003) 4499–4505.