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Conformational substates and dynamic properties of carbonmonoxy hemoglobin

Antonio Cupane[a,*], Maurizio Leone[a], Valeria Militello[b]

[a]*Istituto Nazionale per la Fisica della Materia and Dipartimento di Scienze Fisiche ed Astronomiche, Università di Palermo, Via Archirafi, 36, 90123 Palermo, Italy*

[b]*Istituto Nazionale per la Fisica della Materia and Dipartimento di Medicina Sperimentale, Università di Palermo, Corso Tukory, 129, 90134 Palermo, Italy*

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Abstract

Heme pocket dynamics of human carbonmonoxy hemoglobin (HbCO) is studied by Fourier transform infrared spectroscopy. The CO stretching band at various temperatures in the interval 300–10 K is analyzed in terms of three taxonomic A substates; however, in HbCO the band attributed to the A_1 taxonomic substate accounts for $\approx 90\%$ of the total intensity in the pH range 8.8–4.5. Two different regimes as a function of temperature are observed: below 160 K, the peak frequency and the bandwidth of the A_1 band have constant values whereas, above this temperature, a linear temperature dependence is observed, suggesting the occurrence of transitions between statistical substates within the A_1 taxonomic substate in this protein. The relationship between the heme pocket dynamics (as monitored by the thermal behavior of the CO stretching band), the overall dynamic properties of the protein matrix (as monitored by the thermal behavior of Amide II and Amide I′ bands) and the glass transition of the solvent (as monitored by the thermal behavior of the bending band of water) is also investigated. From this analysis, we derive the picture of a very soft heme pocket of hemoglobin characterized by rather large anharmonic terms and strongly coupled to the dynamic properties of the solvent.

Keywords: FTIR spectroscopy; Protein dynamics; CO stretching band; Amide II band; Amide I′ band; Protein–solvent coupling

1. Introduction

An indepth understanding of protein stability, dynamics and function requires to consider these macromolecules as complex physical objects [1]. The inherent complexity of proteins arises from the fact that they are systems composed by many atoms or groups of atoms that interact, in the absence of periodicity, through a variety of seldom mutually contrasting interactions (e.g. hydrogen bonding, van der Waals forces, electrostatic interactions) no one of which is dominating. In this context, frustration arises, and as a consequence the conformational energy landscape of a protein is not characterized by a single, well defined, minimum but rather by several local minima, corresponding to the same overall 'native' structure

*Corresponding author. Tel.: +39-91-6234221; fax: +39-91-6162461.

E-mail address: cupane@fisica.unipa.it (A. Cupane).

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but differing in structural details, that have been called 'conformational substates' [2,3]. In more precise although still qualitative words, the overall shape of the energy hypersurface is that of a steep well (corresponding to the thermodynamically stable 'native' structure); however, superimposed to the overall shape a rugged structure exists, so that the energy landscape assumes a complex aspect, characterized by many almost iso-energetic local minima separated by energy barriers. The elucidation of the energy landscape of proteins has recently become one of the main goals of biophysical research.

Much effort has been dedicated, in the past, to the study of the energy landscape of carbonmonoxy myoglobin (MbCO). For this molecule, FTIR spectroscopy has proven to be a very useful technique, in that the infrared spectrum of MbCO shows multiple (three) stretch bands of bound CO [4,5], which have been assigned to the three different taxonomic substates A_0, A_1 and A_3 [6,7]. Although the structural assignment of A substates in MbCO has been subject to some controversy [7,8], it is now widely accepted that they arise from protein conformations having different electrostatic interactions between heme–CO moiety and polar residues lining the heme pocket [9–11], therefore modulating the iron–CO π back-bonding. Experiments using mutant proteins [12–14] or as a function of pH [15], theoretical calculations [16] and X-ray crystallographic studies [17] have helped in clarifying structural differences between A substates. Measurements of CO rebinding after flash photolysis at cryogenic temperatures to single A substates have evidenced non-exponential kinetics [5,18], i.e. the existence of statistical substates within taxonomic substates. Frauenfelder et al. [19] have suggested a hierarchical organization of substates in several tiers.

Much less is known about conformational substates of the carbonmonoxy derivative of human hemoglobin (HbCO). Infrared spectra at room temperature have shown the existence of multiple CO stretch bands also for this molecule [20,21]. At pH 7.4, in water/buffer solution, a major band at approximately 1952 cm^{-1} accounts for $\approx$95% of the total intensity while other bands account for only $\approx$5% [20]. However, the characterization of eventual statistical substates is lacking, as well as information on the possible hierarchical organization of the HbCO energy landscape.

In this work, we use FTIR spectroscopy in the temperature interval 300–10 K to address the above problem. The rationale of our approach is to exploit the temperature dependence of the CO stretching band, and in particular of the peak frequency and width, to obtain information on the energy landscape and dynamic properties of HbCO.

In the literature, several models have been proposed to rationalize the temperature dependence of infrared or Raman bands. A first class of models considers the vibrational dephasing resulting from anharmonic coupling of the, essentially homogeneous, band with a low frequency mode; the model predicts 'hyperbolic cotangent-like' shifts of band peak frequency and width [22] from which the frequency of the soft mode and the magnitude of anharmonic coupling can be estimated. A model of this kind has been developed by Shelby et al. [23] and by Marks et al. [24] (see also the more recent work by Bitler and Stavrov [25]) and has been applied by Ondrias et al. [26] to take into account the temperature dependence of the Fe–His stretching band in frozen samples of deoxy hemoglobin. In that case, a smooth peak frequency increase from $\approx$215 cm^{-1} at 270 K to $\approx$230 cm^{-1} at 10 K was observed, together with an analogous smooth decrease in bandwidth. The data were interpreted in terms of vibrational dephasing resulting from anharmonic coupling of the iron—proximal histidine stretch to a low frequency mode; an activation energy (i.e. energy of a quantum of the anharmonically coupled low frequency mode) of approximately 200 cm^{-1} was found, together with strong anharmonic coupling. A model of this kind (although somewhat modified) has been recently applied by Kaposi et al. [27] to analyze the temperature dependence of the infrared absorption spectra of carbonmonoxy horseradish peroxidase.

A different approach has been used by Nienhaus et al. [15] to account for the pH dependent peak frequency shifts observed for the CO stretching band of MbCO. Their model considers temperature dependent protonation/deprotonation of heme

pocket residues having p*K* values approximately 6 and interprets the observed shifts as due to the changing electrostatic environment of the bound CO molecule.

A still different approach considers the effect of protein conformational substates on the largely inhomogeneous spectral bands (see e.g. Ref. [18] and references therein). Within this approach the peak frequency and width of the band reflect the average spectral properties of an heterogeneous protein ensemble. At low temperatures, where each protein is 'frozen' in its particular substate and interconversion among substates is inhibited, temperature independent peak frequency and width values are expected; at sufficiently high temperatures, where substates interconversion is permitted, the thermal behavior of the band profile is expected to reflect the temperature dependence of substates population. The role played by anharmonicity in this approach should be noted; in fact, anharmonic motions are needed in order to have substates interconversion.

In our experiment, the thermal behavior of the infrared spectrum of HbCO clearly shows two different regimes: below 160 K, the peak frequency and the bandwidth of the A_1 band have constant values whereas, above this temperature, a linear temperature dependence is observed. We interpret this result in terms of statistical conformational substates of tier 1 within the taxonomic substate A_1. Substates interconversion, which is inhibited at temperatures lower than 160 K, becomes possible at temperatures above the solvent glass transition: it appears therefore strongly coupled to the solvent dynamics, as monitored by the thermal behavior of the water bending band. At variance, the protein backbone dynamics, as monitored by the amide II and I′ bands, exhibits a smooth temperature dependence and appears weakly coupled to solvent dynamics.

2. Materials and methods

2.1. Samples

Hemoglobin was prepared from the blood of a single healthy individual as already described [28]. Stocks of approximately 10% by weight concentration were stored in liquid nitrogen under CO form. An appropriate amount was thawed immediately before the experiment; the protein was first concentrated to approximately 26% by weight and then diluted with glycerol and buffer. Final samples were 65% v/v glycerol water solutions containing $\approx$6 mM (in heme) protein and 0.1 M phosphate buffer pH 7. The sample was equilibrated with CO by gently stirring under CO atmosphere for approximately 15 min; a few grains of sodium dithionite were also added to ensure full reduction. The high protein concentration used, and the consideration that dimer–tetramer association constant in water–alcohol solutions is even larger than in pure water [29], makes us confident that HbCO in our sample is essentially in tetrameric form at all temperatures. Measurements at various pH values were performed by using 0.15 M borate buffer (pH 8.8) and 0.1 M succinate buffer (pH 4.5); no dithionite was used for these experiments.

A sample in 65% glycerolD_8–D_2O was also prepared, in order to study the protein Amide I′ band. To this purpose, hemoglobin was deuterated by first diluting the protein stock with D_2O (Euroisotope, Grenoble, France, purity > 99.8%) and then re-concentrating; this procedure was repeated several times until a final D_2O concentration larger than 98% was obtained.

2.2. FTIR spectroscopy

FTIR spectra were measured using a Bio-Rad FTS-40A spectrophotometer equipped with a PbS detector and an Oxford Microstat^{He} dewar for measurements in the temperature range 300–10 K. The temperature was checked by an Oxford ITC-503 temperature controller. Samples were placed in an interchangeable precision amalgamated cell mounting CaF_2 windows and a 0.025 mm spacer. At each temperature the single beam spectrum in 1000–4000 cm^{-1} wavenumbers range was measured with 300 scans at 1 cm^{-1} resolution. In a separate experiment, the single beam spectra of each solvent (65% v/v glycerol–water solution with 0.1 M of the relative buffer) were measured at the same temperatures. The sample and solvent absorption spectra were calculated with respect to

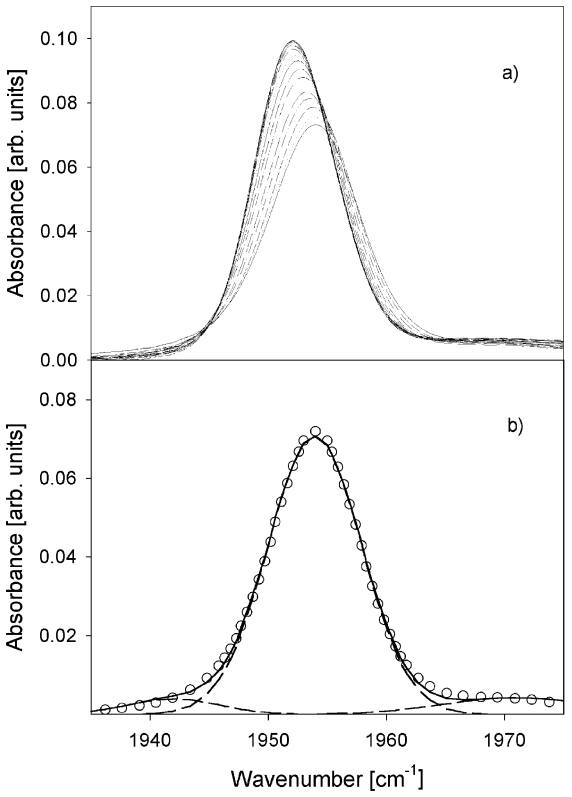

Fig. 1. (a) Infrared absorption spectra of HbCO in the CO stretching region at various temperatures in the range 300 –10 K; (b) deconvolution of the spectrum at 300 K in terms of Gaussian components. The circles are the experimental data, the dashed lines represent the individual components and the continuous line the overall calculated band profile. Fittings of analogous or better quality are obtained at all the temperatures.

the single beam of empty cell; at each temperature, solvent spectrum was subtracted from the sample spectrum after suitable normalization.

3. Results and discussion

Fig. 1a shows the infrared spectrum of HbCO in the C–O stretch region at various temperatures in the range 15–300 K. The peak frequency of the spectrum shifts to lower energies as the temperature is lowered, while the bandwidth decreases and the integrated intensity increases; spectral differences between α and β chains (reported to be approximately 1 cm^{-1} at room temperature [30]) remain undetected even at low temperatures.

Fig. 1b shows a fitting of the 300 K spectrum in terms of Gaussian components. Potter et al. [20] have shown that this spectrum can be fit with four symmetric bands; however, the lowest frequency component at approximately 1930 cm^{-1} accounts for only 1% of the total intensity. We therefore used three Gaussian components: one at approximately 1942 cm^{-1} (that has to be considered as the sum of bands I and II of Potter et al. [20]), another at approximately 1954 cm^{-1} and the last one at approximately 1970 cm^{-1}. A fit with Voigtian components (convolutions of Gaussians and Lorentzians) gave essentially identical results; however, the ratio of the Gaussian over the Lorentzian width was approximately 3–4 at all temperatures and for all components. In order to avoid using a large number of poorly defined fitting parameters we have chosen to analyze the spectra in terms of Gaussian components. The fractional integrated intensities of the three bands at room temperature are approximately 5, 88 and 7%, respectively, in agreement with Potter et al. [20]; the slightly larger intensity of the component at 1970 cm^{-1} may be attributed to the lower pH value of our experiment and/or to the different solvent composition. Data in Fig. 1 also show that at any temperature within the range studied the band at 1954 cm^{-1} accounts for approximately 90% of the total intensity while the two side bands, together, for only approximately 10%: the population change between substates A_1 and A_0 seen in MbCO as the temperature is lowered [6] is not observed for HbCO. For this reason, in the following we will report only on the temperature dependence of the spectral parameters relative to the conformer responsible for the band at approximately 1954 cm^{-1} that, for the sake of conciseness, will be called substate A_1.

The temperature dependence of the peak frequency (parameter ν_0) and of the bandwidth (parameter σ^2) are shown in Fig. 2a and b, respectively. Two different regimes are clearly identified in Fig. 2: one at temperatures lower than 160 K, where ν_0 and σ^2 are essentially temperature independent, and the other at temperatures higher than 160 K, where they vary almost linearly with temperature.

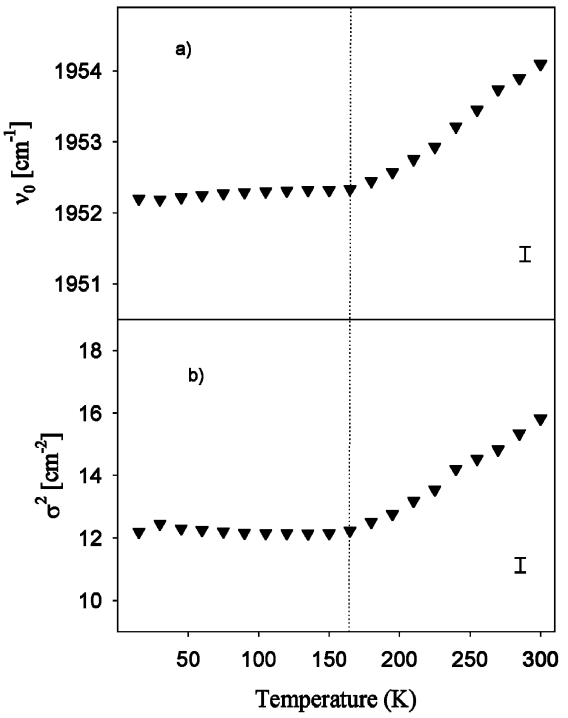

Fig. 2. Temperature dependence of (a) the peak frequency, ν_0; and (b) the bandwidth, σ^2, relative to substate A_1 of Hb–CO. Typical error bars are shown.

We tried to rationalize the data reported in Fig. 2 within the framework of the various approaches described in the introduction. Analysis in terms of the 'anharmonic coupling' model as described in Refs. [23,24,26] shows that the predictions of the model for the temperature dependence of the band peak frequency are not obeyed in the whole temperature range; moreover, activation energy of approximately 700 cm^{-1} and strong anharmonic coupling is found from the high temperature fittings. In the resonance Raman spectra of HbCO in the frequency region approximately 700 cm^{-1} only the well known ν_7 band is found at approximately 676 cm^{-1} and is attributed to a totally symmetric (A_{1g} type, in D_{4h} symmetry) breathing mode of the porphyrin ring [31,32]; strong coupling of ν_7 to the C–O stretch is highly unlikely because of symmetry considerations.

As mentioned in the Introduction, Nienhaus et al. [15] have recently developed an alternative model to account for the pH dependence of the infrared spectra of sperm whale MbCO. The pH-dependent spectral changes observed in MbCO were unambiguously assigned to the protonation of the two histidines H97 (FG3) and H64 (E7). In particular, the ~ 2 cm^{-1} blue shift of the A_1 and A_3 lines observed with decreasing pH was clearly assigned to the protonation of histidine H97 (FG3) that, in sperm whale MbCO, is stacked against the heme on the proximal side. In human hemoglobin, the residue in position FG3 is a leucine, both in the α and in the β chains; moreover, a red shift of the A_1 line is observed as the temperature is lowered; this rules out an involvement of this residue in the observed effect. However, a temperature dependent protonation/deprotonation of a heme pocket residue having a pK value approximately 6–7 could in principle be responsible for the effects reported in Fig. 2. To investigate this point we performed experiments at various pH values in the range 4.5–8.8. The temperature dependence of peak frequency at various pH values is reported in Fig. 3, while values of the quantity $d\nu_0/dT$, calculated at various pHs from a linear fit to the high temperature behavior, are reported in Table 1. Data in Fig. 3 and Table 1 show that even at pH 8.8 and 4.5 a marked red shift, almost linear, of the A_1 line is observed by lowering the temperature at $T > 160$, whereas, at $T < 160$ K, parameter ν_0 remains almost constant.

From the above considerations we conclude that vibrational dephasing and temperature dependent protonation/deprotonation do not contribute significantly to the effects reported in Fig. 2 and we examine an explanation in terms of protein conformational substates. Following Ansari et al. [6] we take the rather large residual bandwidth at low temperatures as evidence for the presence of a 'statistical' population of frozen conformational substates (substates of tier 1) within the 'taxonomic' substate A_1; at variance with the taxonomic substates, these substates cannot be sorted out individually and for this reason they are called statistical substates [3]. At temperatures lower than 160 K, the energy barriers between these substates are much higher than K_BT and the transition rates are very slow, so that interconversion among them is not observed in the time scale of our experiment

and each protein is frozen in a particular substate: temperature independent ν_0 and σ^2 values simply reflect the ensemble averaged spectral properties of an heterogeneous protein population 'frozen' at $T \approx 160$ K. At temperatures higher than 160 K transition rates between statistical substates of tier 1 become low enough so that interconversion is allowed. The rather steep temperature dependence of the transition rates between statistical substates may be due either to temperature dependent barrier heights, or to a non-Arrhenius temperature dependence—e.g. a Ferry-like behavior [33,18]—implying dynamics on a rugged potential well.

To take into account the reported data, we suppose a mapping between the peak frequency of the CO stretching band and some generalized protein conformational coordinate. In view of the current interpretation of the CO stretching frequency of CO bound to myoglobin [9,34], this configurational coordinate can be related to the distance of some polar/charged group of the heme pocket from the heme–CO complex. The remarkable insensitivity of the observed effect to pH in the range 4.5–8.8 rules out a direct involvement of the distal histidine. The charged lysines at position FG2 in the proximal side and at the position E10 in the distal side are, in our opinion, plausible candidates: as the temperature is increased the distance of the above positively charged groups from the bound CO increases because of thermal expansion, iron–CO π back-bonding decreases and ν_{CO} increases. The role of charged residues of the proximal heme pocket in shifting the peak frequency of the A_1 substate of MbCO has recently been pointed out by Muller et al. [15].

Table 1
Values of the quantity $d\nu_0/dT$ as a function of pH

pH[a]	$d\nu_0/dT$ ($cm^{-1}\,K^{-1}$)[b]
8.8	$(1.4\pm0.1)\times10^{-2}$
7.0	$(1.4\pm0.1)\times10^{-2}$
5.5	$(1.2\pm0.1)\times10^{-2}$
4.5	$(1.2\pm0.1)\times10^{-2}$

[a] The estimated uncertainty in the pH values is ±0.1 pH units.

[b] $d\nu_0/dT$ values have been calculated by linear least squares fittings of data in the temperature range 180 –300 K.

To investigate the relation between heme pocket and solvent dynamics, we have measured the temperature dependence of the bending band of water at approximately 1650 cm^{-1}; the thermal behavior of the spectral profile and of the peak

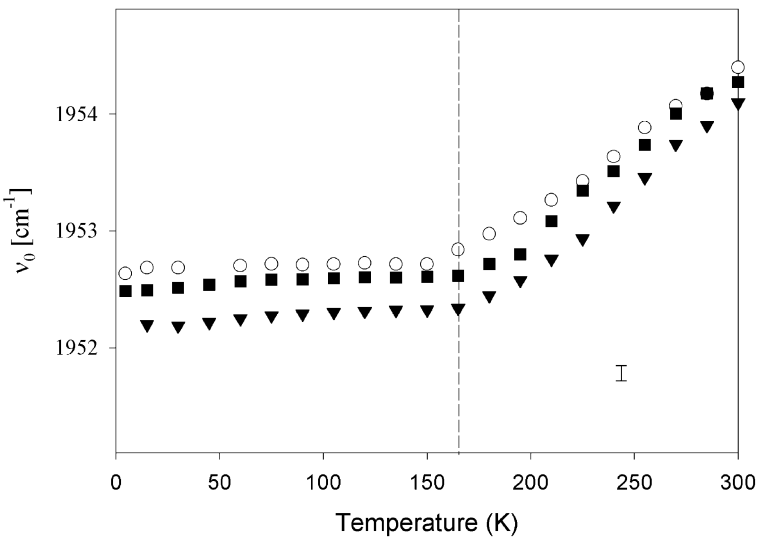

Fig. 3. Temperature dependence of the peak frequency at various pH values. (○) pH 4.5; (■) pH 8.8; (▼) pH 7. A typical error bar is shown.

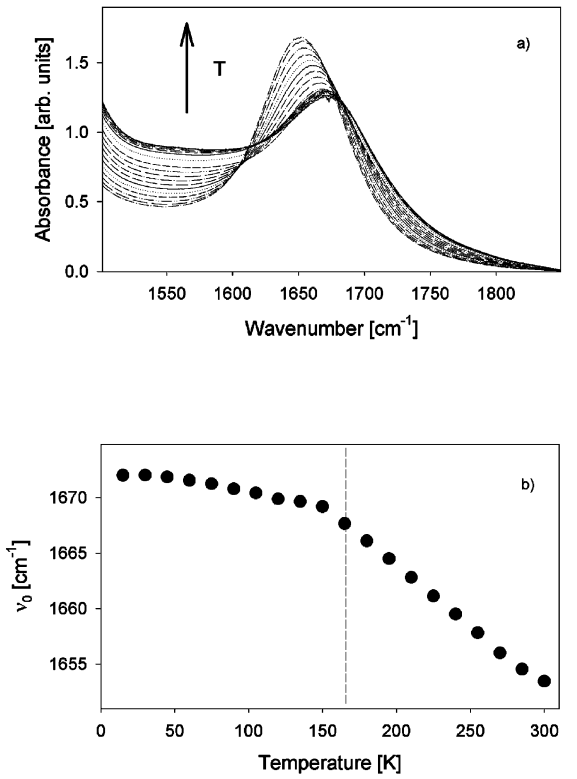

Fig. 4. Temperature dependence of bending band of water: (a) the overall band profile; (b) the peak frequency, ν_0. The arrow in panel (a) indicates the direction of the spectral variations observed upon increasing the temperature.

frequency of this band is reported in Fig. 4. In analogy with data reported by Demmel et al. [35], the occurrence of a 'break' at 150–160 K, i.e. at the solvent glass transition, is clearly observed. The close analogy between data in Figs. 1–4 confirms that the heme pocket dynamics in hemoglobin—as monitored by the stretching band of the bound CO—is indeed intimately related to the solvent dynamics—as monitored by the bending band of water.

Further insight can be obtained by looking at the global protein dynamics as monitored, at the level of secondary structure, by the thermal behavior of the amide II band at ≈ 1550 cm^{-1}. The results are reported in Fig. 5. Surprisingly, a smooth temperature dependence—attributed to the softening of α-helical hydrogen bonds by increasing temperature [35,36]—is observed and no inflection at the solvent glass transition is detected. This is at variance with results from Demmel et al. [35] relative to myoglobin and from Manas et al. [36] relative to parvalbumin. This result indicates that, in hemoglobin, the dynamics of the protein—observed at the level of the α-helical secondary structure—is less coupled to solvent dynamics than in monomeric proteins like myoglobin or parvalbumin, likely due to the tetrameric structure of this protein.

To ascertain whether the behavior reported in Fig. 5 is peculiar of the amide II band or common to other secondary structure marker bands, we have investigated also the temperature dependence of the amide I band. Since in H_2O this band is partially obscured by the bending band of solvent, we have performed experiments with a sample of

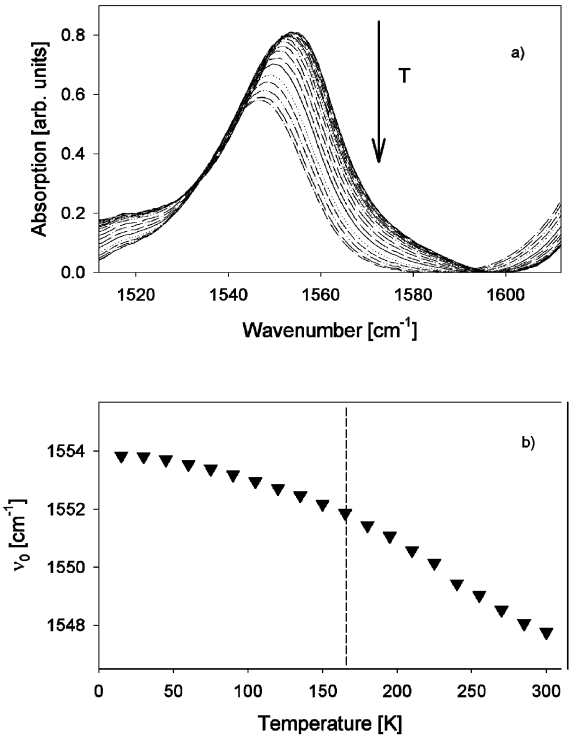

Fig. 5. Temperature dependence of Amide II band of HbCO: (a) the overall band profile; (b) the peak frequency, ν_0. The arrow in panel (a) indicates the direction of the spectral variations observed upon increasing the temperature.

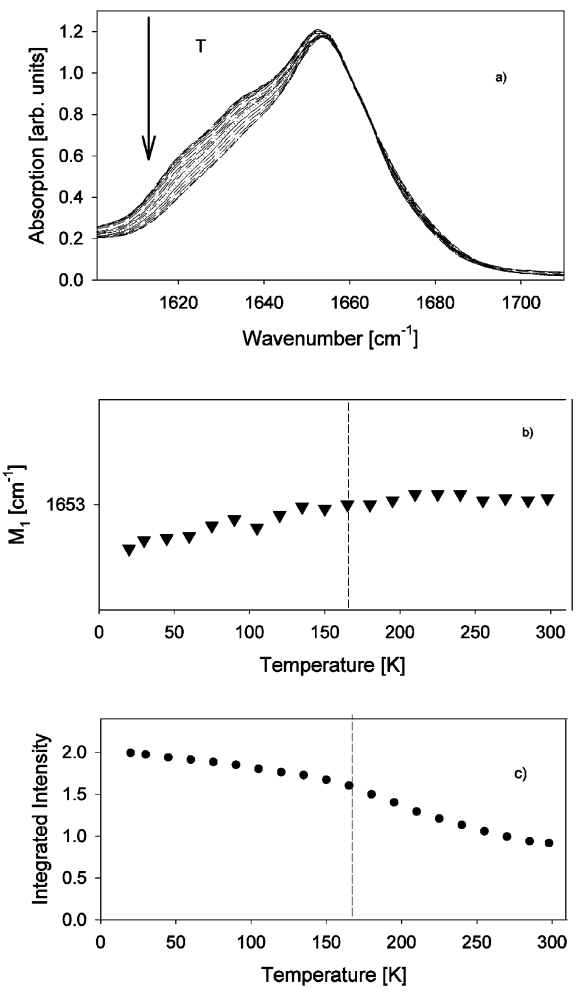

Fig. 6. Temperature dependence of Amide I′ band of HbCO in glycerolD_8–D_2O solution: (a) the overall band profile; (b) the peak frequency (first moment) of the band at $\approx$1650 cm^{-1}; (c) the integrated intensity of the band at $\approx$1630 cm^{-1}. The arrow in panel (a) indicates the direction of the spectral variations observed upon increasing the temperature.

deuterated hemoglobin in glycerolD_8–D_2O solution. The temperature dependence of the amide I′ band is reported in Fig. 6a. As it is well known [36–39], the amide I′ band of hemoglobin consists of two main sub-bands, one at approximately 1650 cm^{-1} and the other at approximately 1630 cm^{-1}, that are attributed to buried and solvent-exposed protein α-helices, respectively. The thermal behavior of the two sub-bands is strikingly different. In fact, sub-band at approximately 1650 cm^{-1} has a weak dependence upon temperature and does not show any break or inflection at the solvent glass transition; conversely, sub-band at approximately 1630 cm^{-1} has a marked temperature dependence (consisting mainly in integrated intensity increase and peak frequency red shift) which is weakly coupled to the solvent dynamics. This is more clearly shown in Fig. 6b and c, where we report the peak frequency (first moment) of the 1650 cm^{-1} band and the integrated intensity of the 1630 cm^{-1} as a function of temperature, respectively.

We think that the reported results clearly indicate that different dynamic properties are measured in different parts of the protein. In particular, the heme pocket dynamics in hemoglobin, as measured by the temperature dependence of the CO stretching band, appears linked to the onset of interconversion between statistical conformational substates within the taxonomic substate A_1, thus highlighting the hierarchical organization of the energy landscape in hemoglobin. Moreover, heme pocket dynamics in hemoglobin is strongly coupled (‘slaved’, [6]) to the solvent dynamics—much like in myoglobin [6,40]—likely due to the fact that heme pockets are located near the surface of the hemoglobin molecule. At variance, overall protein dynamics, as measured at the level of secondary structure by the temperature dependence of the amide II band and of the amide I′ sub-band attributed to buried protein α-helices, is not influenced by the solvent glass transition; this behavior can be attributed to the tetrameric structure of the hemoglobin molecule. Not surprisingly, weak coupling with solvent dynamics is observed for the amide I′ sub-band attributed to solvent-exposed protein α-helices

4. Conclusions

In this work we have characterized the energy landscape of the heme pocket in HbCO in terms of taxonomic conformational substates (substates of tier 0) and of statistical conformational substates (substates of tier 1 or lower). Investigation as a function of temperature has clearly shown the presence of two different regimes. At temperatures higher than 160 K, interconversion between statis-

tical substates occurs and the system can reach thermodynamic equilibrium. In this regime the peak frequency and half width of the infrared band relative to substate A_1 display linear temperature dependence. At temperatures lower than 160 K interconversion between substates is inhibited: the spectral parameters of the A_1 band become temperature independent and their values reflect the ensemble averaged properties of the heterogeneous population of statistical substates of tier 1. The fact that the transition temperature is close to the glass transition of the solvent matrix suggests that heme pocket–solvent interactions are relevant to this effect, and indeed a close correlation with solvent dynamics—as measured by the temperature dependence of the water bending band—is observed. At variance, protein dynamics—as measured at the level of secondary structure by the temperature dependence of the amide II and I′ bands—is barely influenced by the solvent glass transition. This highlights the fact that different dynamic properties are measured in different parts of the protein and indicates the necessity of monitoring the dynamics at several different structural levels and using various experimental techniques, to reach a more complete description of protein dynamics.

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