Eur Biophys J (1993) 21, 385–391

European Biophysics Journal

Protein dynamics: conformational disorder, vibrational coupling and anharmonicity in deoxy-hemoglobin and myoglobin

Antonio Cupane, Maurizio Leone, Eugenio Vitrano

Istituto di Fisica dell' Università and GNSM-INFM, Via Archirafi 36, I-90123 Palermo, Italy

Received: 24 August 1992 / Accepted in revised form: 21 October 1992

Abstract. In this work we study the temperature dependence of the Soret band lineshape of deoxymyoglobin and deoxyhemoglobin, in the range 300–20 K. To fit the measured spectra we use an approach originally proposed by Champion and coworkers (Srajer et al. 1986; Srajer and Champion 1991). The band profile is modelled as a Voigt function that accounts for the coupling with low frequency vibrational modes, whereas the coupling with high frequency modes is responsible for the vibronic structure of the spectra. Moreover, owing to the position of the iron atom out of the mean heme plane, inhomogeneous broadening brings about a non-Gaussian distribution of 0-0 electronic transition frequencies. The reported analysis enables us to isolate the various contributions to the overall bandwidth, and their temperature dependence points out the relevance of low frequency vibrations and of large scale anharmonic motions starting at temperatures higher than 170 K. Information on the mean iron-heme plane distance and on its temperature dependence, as well as on the heme pocket conformational disorder, is also obtained.

Key words: Heme proteins – Optical spectroscopy – Dynamic properties

Introduction

The optical absorption spectra of metallo proteins depend on temperature and their thermal behavior (that is due to the interaction of the electronic transition with the nearby nuclei) contains information on the dynamic properties of the protein matrix surrounding the chromophore (Cordone et al. 1986, 1988; Cupane et al. 1988, 1990; Di Iorio et al. 1991; Leone et al. 1987, 1992).

In the case of the Soret band of heme proteins, owing to the large extinction coefficient, electron-vibration interactions can be fairly well described within the Franck-Condon approximation, so that a non-heuristic deconvolution of the measured spectra at various temperatures can be obtained (Srajer et al. 1986; Shomacker and Champion 1986; Di Pace et al. 1992). In this way the various contributions to the overall bandwidth can be singled out and their temperature dependence can be studied.

Various line broadening mechanisms are relevant in the system studied; these are: homogeneous broadening due to the non-radiative decay of the excited electronic state, coupling with high frequency nuclear vibrations that is responsible for the vibronic structure of the optical absorption spectra, Gaussian broadening due to the coupling with a "bath" of low frequency motions, inhomogeneous broadening due to the conformational heterogeneity. This last effect can be modelled with a distribution of the purely electronic (0-0) transition frequencies: a Gaussian distribution is suitable for the CO derivatives (Schomacker and Champion 1986; Di Pace et al. 1992), whereas in the case of deoxy derivatives the position of the iron atom out of the mean heme plane brings about a non-Gaussian distribution that is responsible for the marked asymmetry of the band (Srajer et al. 1986; Srajer and Champion 1991).

In previous studies from our group on the temperature dependence of the optical spectra of liganded and unliganded hemoglobin and myoglobin (Cordone et al. 1986; Cupane et al. 1988; Leone et al. 1987), the Soret band was heuristically deconvoluted into Gaussian components, so that the various line broadening mechanisms could not be singled out.

In this work we apply the approach outlined above to analyze the temperature dependence of the Soret band of Hb and of SWMb, in the range 300–20 K. The temperature dependence of the iron-heme plane geometry and of its conformational disorder are demonstrated in this

Correspondence to: A. Cupane

Abbreviations: Co: Carbon monoxide; Hb: human deoxyhemoglobin A; HbCO: human carbonmonoxyhemoglobin A; SWMb: spermwhale deoxymyoglobin; SWMbCO: spermwhale carbonmonoxymyoglobin; HbO_2: human oxyhemoglobin A; $SWMb^{3+}$-H_2O: spermwhale aquometmyoglobin

study. Moreover, as for CO derivatives (Di Pace et al. 1992), the onset of anharmonic motions at about 170 K is evident. The study of two different proteins and comparison of the results with analogous ones, relative to CO derivatives, enables us to investigate the dependence of the relevant parameters upon protein structure and the effect of the bound ligand on the dynamic properties of the heme pocket.

Materials and methods

SWMb was purchased from Sigma and used without further purification. Appropriate amounts of lyophilized ferric protein were dissolved in 0.1 M phosphate buffer pH 7.0 and centrifuged to remove any precipitate.

Human Hb was prepared from the blood of a single donor, as already described (Cordone et al. 1979). Concentrated stocks of Hb were stored in the oxy form at 77 K and melted immediately before the use.

Approximately 10 ml of protein solution (0.1 M phosphate buffer pH 7.0 in water at room temperature; 65% v/v glycerol and approximately 10^{-5} M HbO_2 or $SWMb^{3+}$-H_2O) were thoroughly deoxygenated in a tonometer. Reduction of the protein was performed by adding 50 µl of a previously deoxygenated, 2% by weight, sodium dithionite solution; complete reduction was checked spectrophotometrically. The samples were then anaerobically transferred from the tonometer to the cuvette and to the dewar.

Glycerol (Fluka, Buchs, Switzerland) was analytical grade and was used without further purification. Spectra (500–300 mm) were taken with a Jasco Uvidec 650 spectrophotometer (bandwidth = 0.4 nm; time constant = 1 s; scan speed = 40 nm/min). The experimental setup and method for optical measurements in the temperature range 300–20 K have been described by Cordone et al. (1986). Metacrylate cuvettes (Kartell) of 1 cm path length were used. We stress that our samples remained homogeneous and transparent at all temperatures and that cracks were not observed. The absence of scattering due to sample cracking results in a remarkable stability of the baseline with temperature and makes possible the kind of spectral analysis reported. The baseline (cuvette + solvent + buffer) measured at room temperature was subtracted from each spectrum. At the concentrations used, the absorption due to dithionite becomes relevant at wavelengths lower than $\simeq$370 nm. For this reason the spectral analysis was limited to the wavelength range 500–380 nm; in this range the baseline does not depend on temperature.

Absorption spectra were recorded at 0.4 nm intervals. Deconvolutions were performed on a Microway I860 add-on board for an IBM-PC using a non-linear least-squares algorithm; errors on fitted parameters were calculated by inversion of the curvature matrix (Bevington 1969), within the approximation of parabolic χ^2 surface around the minimum and correspond to 67% confidence limits.

To isolate the Soret band from the background generated by other electronic transitions, the absorbance at 480 nm has been brought to 0.02 for all the spectra and a Gaussian component centered at 27 000 cm^{-1} has been added in the analysis, to take into account contributions due to the N band.

To obtain an analytical expression for the Soret band profile at various temperatures, we consider a single electronic transition coupled with several harmonic, Franck-Condon active, vibrational modes and we treat our system within the Born-Oppenheimer and Condon approximation.

It has been shown (Di Pace et al. 1992) that the absorbance at frequency ν can be written as:

$$A(\nu) = M\,\nu \sum_{m_1,\ldots,m_{N_h}}^{\infty} \prod_{i}^{N_h} \frac{S_i^{m_i}\, e^{-S_i}}{m_i!} \times \frac{\Gamma}{\left[\nu - \nu_0 - \sum_{i}^{N_h} m_i R_i \nu_i(g)\right]^2 + \Gamma^2} \otimes \frac{1}{\sigma} e^{-\frac{\nu^2}{2\sigma^2}} \quad (1)$$

In (1) M is a constant proportional to the square of the electric dipole moment; Γ is a damping factor related to the finite lifetime of the excited state (homogeneous broadening); the product extends to all "high frequency" vibrational modes coupled to the electronic transition and the summations to their occupation numbers; $\nu_i(g)$, S_i and R_i are the frequency, linear coupling constant and quadratic coupling constant of the i-th "high frequency" vibrational mode ("high frequency" means $h\nu_i \gg K_B T$). The symbol $\otimes$ indicates the convolution operator.

In the derivation of (1), the coupling of the electronic transition with low frequency modes (frequency smaller than the observed bandwidth) is treated within the "short times approximation" (Chan and Page 1983); this brings about the convolution with a Gaussian lineshape and contributes temperature dependent terms to the linewidth and peak position of the band. By considering a bath of low frequency modes, the parameters σ and ν_0 in (1) can be expressed as:

$$\sigma^2 = NSR^2 \langle\nu\rangle^2 \coth(h\langle\nu\rangle/2K_B T) \quad (2)$$

$$\nu_0 = \nu_{00} - 1/4\,N\langle\nu\rangle(1-R)\coth(h\langle\nu\rangle/2K_B T) + C \quad (3)$$

where $\langle\nu\rangle$, S and R are the effective frequency, linear and quadratic coupling constants of the low frequency bath, N is the number of the soft modes, ν_{00} is the frequency of the purely electronic (0-0) transition, and C takes into account other temperature independent contributions to the peak positions.

Further contributions to the spectral linewidth are given by inhomogeneous effects arising from different conformational substates and heme group environments (Frauenfelder et al. 1988; Ormos et al. 1990). If such conformational heterogeneity is present, one has to perform averages over all spectrally different species; assuming that the variance of the various parameters that describe the electron-vibrations coupling due to conformational heterogeneity is small, one can replace them with average values and inhomogeneous broadening becomes evident. In the case of CO derivatives of various hemeproteins, such broadening has been modelled as a Gaussian distri-

bution of ν_{00} frequencies that contributes a further term to the width of the Gaussian component of the Voigtian in (1).

In the case of deoxy hemeproteins, however, it has been suggested that the disorder in the position and orientation of the iron atom with respect to the heme plane influences the energy of the $\pi \rightarrow \pi^*$ electronic transition responsible for the Soret band and that therefore the $\pi \rightarrow \pi^*$ excitation frequency can be written as:

$$\nu_{00} = \nu_{00}\,(Q=0) + b\,Q^2 \tag{4}$$

where Q represents a generalized iron coordinate that describes both the out of plane displacement and other angular coordinates such as the histidine tilt and the azimuthal orientation of the vector connecting the iron with the histidine nitrogen (for a deeper discussion of (4) see Srajer et al. (1986) and Srajer and Champion (1991)). The parameter b is a "force constant" that accounts for the dependence of the $\pi \rightarrow \pi^*$ transition energy upon iron coordinates and reflects the electronic properties of the iron-porphyrin system; therefore it is not expected to depend upon temperature or protein structure (at least for proteins with similar heme pocket structure). In what follows (see "Results and discussion") we will assume that parameter b is equal for Hb and SWMb and temperature independent. Assuming that the coordinate Q is statistically distributed, i.e. that

$$P\,(Q) \propto \mathrm{Exp}\,[-(Q-Q_0)^2/2\,\delta^2] \tag{5}$$

where Q_0 is the mean coordinate position (i.e. the mean out of plane iron displacement) and δ the width of the distribution, a non-Gaussian distribution of transition frequencies is immediately found. The analytical expression suitable for the analysis of the Soret band lineshape therefore becomes:

$$A\,(\nu) = V\,(\nu) \otimes \frac{1}{2\delta\sqrt{(\nu-\nu_0)\,2\pi\,b}} \cdot \left[\exp\left(-\frac{[(\nu-\nu_0)^{1/2}+Q_0\sqrt{b}]^2}{2\,b\,\delta^2}\right) + \exp\left(-\frac{[(\nu-\nu_0)^{1/2}-Q_0\sqrt{b}]^2}{2\,b\,\delta^2}\right)\right] \tag{6}$$

where $V\,(\nu)$ is given by the second member of (1).

Fittings of the experimental spectra are performed with (6). The fitting parameters are M, Γ, S_i, σ, ν_0, $Q_0\sqrt{b}$ and $\delta\sqrt{b}$. ν_i values relative to high frequency modes are known from resonance Raman (RR) spectra reported in the literature (Bangcharoenpaurpong et al. 1984; Spiro 1983; Morikis et al. 1991); only the most coupled modes, corresponding to $\nu = 370\ \mathrm{cm}^{-1}$, $\nu = 674\ \mathrm{cm}^{-1}$ and $\nu = 1\,357\ \mathrm{cm}^{-1}$, are considered since the other less coupled modes do not contribute significantly to the spectra. In analogy with what has been done for CO derivatives, we also tried fittings in which a further mode at $\nu = 1\,100\ \mathrm{cm}^{-1}$ was considered; the linear coupling strength, however, was found to be less than 0.01, and for this reason this mode was not considered. $674\ \mathrm{cm}^{-1}$ and $1\,357\ \mathrm{cm}^{-1}$ correspond to very sharp lines in the RR spectra of hemeproteins and are thought to arise from in plane vibrational modes of the heme group ($\nu_7 \equiv \delta\,C_aC_bN$ and $\nu_4 \equiv C_aN$, respectively) (Spiro 1983). $370\ \mathrm{cm}^{-1}$, on the contrary, is an "average effective" frequency accounting for a spectral region characterized by several quasi degenerate peaks; both in plane and out of plane modes contribute to this spectral region. In view of the rather large Γ values obtained, contributions to the S value of the $370\ \mathrm{cm}^{-1}$ mode coming from the coupling of the Soret band with the Fe-N_{His} stretch at about $220\ \mathrm{cm}^{-1}$ cannot be excluded. S_i values are rather well determined, at low temperature, from the vibronic structure of the band (see Fig. 2); to avoid fitting ambiguities arising from broadening of the band and consequent lack of a clearly resolved vibronic structure at high temperatures, parameters S_i were fixed at their low temperature value. Temperature independent S_i values are in agreement with what has been previously found for the CO derivatives of human Hb, SWMb and isolated α and β chains (Di Pace et al. 1992) and for cytochrome c (Schomacker and Champion 1989).

In the fitting procedure the Voigtians appearing in (6) are numerically evaluated as the real part of the complex error function (Gautschi 1970). We use a particular algorithm that calculates the real part of the complex error function via a continuous fraction whose convergence depends on the ratio of parameters σ and Γ (the greater σ/Γ, the slower the convergence); in our cases σ/Γ values range from ≈ 0.5 at $T = 20$ K to ≈ 2 at $T = 300$ K, so that the convergence is amply ensured by truncating the fraction at about the 100^{th} step. The further convolution is also performed by numerical methods; the integral, in this case, is calculated with the trapezoidal approximation. We have also studied the convergence of the infinite sum in (1) and (6). For large m_i values the term $S_i^{m_i}\,e^{-S_i}/m_i!$ approaches zero. We neglect those terms of the series corresponding to Voigtians with amplitude less than 0.1% of the 0-0 amplitude. For the cases investigated, S_i values enable us to truncate the sum at m_i never greater than 3.

Results and discussion

Figure 1 shows the low temperature spectra of Hb and SWMb, together with the fitting obtained using (6); the residuals are also shown, on an expanded scale. The fittings are quite satisfactory; we find no evidence of the extra bands observed at 10 K by Champion and coworkers (Srajer and Champion 1991).

Values of the linear coupling constants for the high frequency modes and of the Lorentzian width (Γ) are shown in Table 1. Γ values are almost identical for Hb and SWMb and do not depend on temperature. In particular the abrupt Γ variation observed, for SWMbCO, at the temperature of the solvent glass transition (≈ 180 K) (Di Pace et al. 1992) is not present in SWMb. This fact confirms the suggestion that the effect observed in SWMbCO is to be related to the tilting of the bound CO molecule towards the heme normal (Di Iorio et al. 1991, Di Pace et al. 1992) that occurs when the temperature is lowered

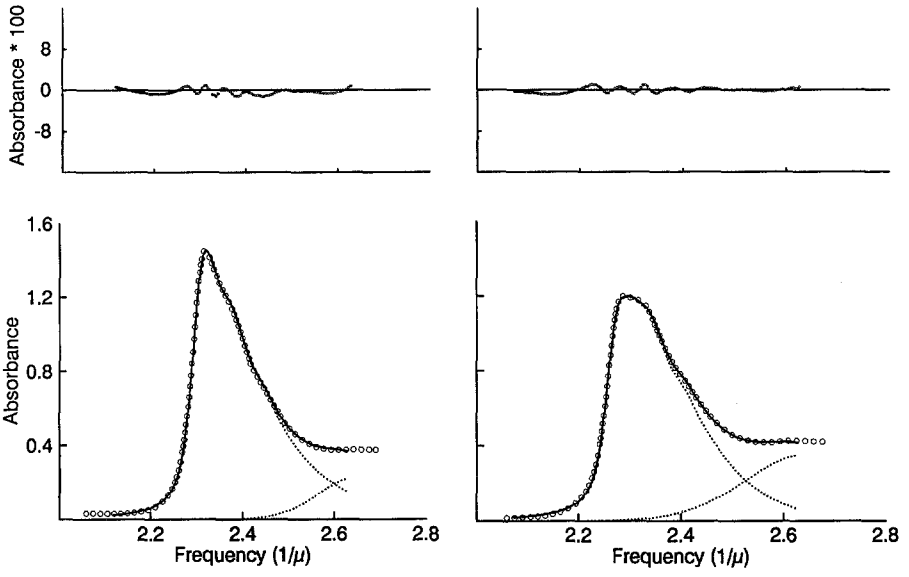

Fig. 1. Spectra of Hb (*left*) and of SWMb (*right*) in 65% glycerol/water at $T=20$ K. Circles are the experimental points; the dotted lines represent fittings in terms of (6) and contributions from N band; the overall synthesized band profile is represented by the continuous lines. For the sake of clarity not all the experimental points have been reported. The residuals are also reported in the upper panels, on an expanded scale

Table 1. Low temperature ($T=20$ K) linear coupling constants of the high frequency modes and Γ values for Hb and SWMb. Analogous data relative to HbCO and SWMbCO, taken from Di Pace et al. (1992), are also reported for comparison

	S_{370}	S_{674}	S_{1100}	S_{1357}	Γ/cm^{-1}
Hb	0.108 ± 0.015	0.208 ± 0.018	<0.01	0.051 ± 0.008	175 ± 9
SWMb	0.321 ± 0.022	0.245 ± 0.020	<0.01	0.095 ± 0.009	180 ± 10
HbCO	0.047 ± 0.009[a]	0.073 ± 0.005[b]	0.016 ± 0.005	0.079 ± 0.004[c]	215 ± 4
SWMbCO	0.116 ± 0.017[a]	0.056 ± 0.010[b]	0.018 ± 0.009	0.092 ± 0.008[c]	211 ± 7

[a] Data for HbCO and SWMbCO refer to the mode at 350 cm^{-1}
[b] Data for HbCO and SWMbCO refer to the mode at 676 cm^{-1}
[c] Data for HbCO and SWMbCO refer to the mode at 1 374 cm^{-1}

below that of the solvent glass transition. It should be noted that the changes in the temperature dependence of peak frequency, linewidth and integrated intensity of the CO stretching bands characteristic of the A_0 and A_1 substates of SWMbCO observed at temperatures where the solvent undergoes the glass transition (Ansari et al. 1987) do not contradict the above suggestion. From Table 1 we note also that S_i values relative to SWMb are larger than those relative to Hb; this effect is also present, although to a much lower extent, in the CO derivatives (Di Pace et al. 1992) and is possibly related to a different puckering of the heme group in the two proteins. S_i and Γ values relative to SWMb can be compared with the analogous ones measured by Bangcharoenpaurpong et al. (1984) and by Srajer et al. (1986). For the mode at 1 357 cm^{-1} our value of 0.095 compares rather well with the value of 0.058 reported by those authors. For the mode at 370 cm^{-1} we find $S_{370}=0.321$ to be compared with $S_{370}=0.02$; it must be remembered, however, that our S_{370} value is the sum of the coupling constants of all the modes in the spectral region around 370 cm^{-1} and can also have contributions from the out-of-plane Fe-His stretching mode at 220 cm^{-1} ($S_{220}=0.16$ and $S_{150}=0.09$ have been reported by the above authors). For the mode at 674 cm^{-1} our value of 0.245 is substantially larger than that (0.035) reported by Bangcharoenpaurpong et al. (1984). In contrast, our Γ value (180 cm^{-1}) is smaller than that (285 cm^{-1}) reported previously (Srajer et al. 1986; Srajer and Champion 1991). It must be stressed that our Γ and S_i values are rather well determined from the almost Lorentzian line shape of the Soret band's red edge and from the low temperature vibronic structure respectively. This is shown in Fig. 2 where we have reported the 20 K spectra of Hb and SWMb after subtraction of the N band and suitable shift of the peak frequency: it is evident that the red edge shape is identical (equal Γ values) whereas the coupling with high frequency modes is stronger for SWMb than for Hb. A possible explanation for the larger Γ values reported by Srajer et al. (1986) and Srajer and Champion (1991) could lie on fitting artifacts arising from light scattering of slightly cracked, and therefore not perfectly transparent, samples.

The σ^2 and ν_0 temperature dependence is reported in Fig. 3. It is evident that (2) and (3) cannot be fitted to σ^2 and ν_0 values in the whole temperature range 20–300 K. Concerning σ^2 values, (2) requires, in the limit $K_B T > h\langle\nu\rangle/2$, that the σ^2 thermal behavior should be given by a straight line whose intercept at $T=0$ K is 0. From Fig. 3 is evident that a straight line tangent to the high temperature experimental points would have a negative intercept at $T=0$ K, and in turn this would clearly imply unphysical negative σ^2 values, arising from inhomogeneous

Table 2. Values of the parameters obtained by fitting the σ^2 and ν_0 behavior in the temperature range 20–160 K in terms of (2) and (3). Analogous data relative to HbCO and SWMbCO, taken from Di Pace et al. (1992), are also reported for comparison

	NS[a]	$\langle\nu\rangle/cm^{-1}$	R[b]	$(\nu_{00}(Q=0)+C)/cm^{-1}$
Hb	0.6±0.1	140±10	0.990±0.001	23 043±2
SWMb	0.7±0.1	110±10	1.02 ±0.004	22 632±2
HbCO	0.5±0.2	170±20	0.990±0.006	23 944±4
SWMbCO	0.3±0.2	180±30	1.01 ±0.002	23 567±4

[a] NS values were obtained under the assumption $R^2 \approx 1$
[b] The R values reported are obtained by assuming the coupling with $N=50$ low frequency modes

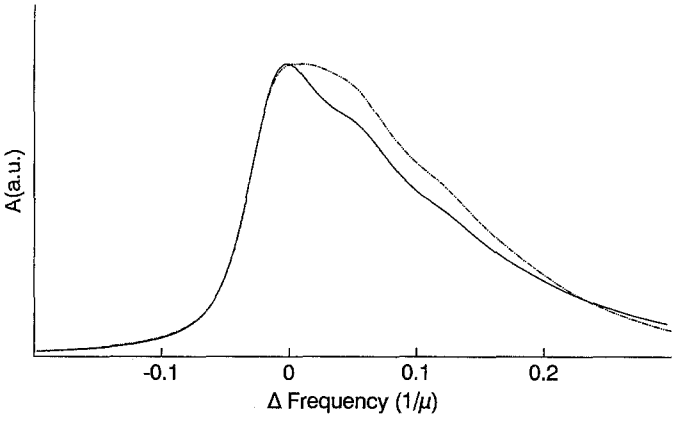

Fig. 2. Soret band of Hb (*continuous line*) and of SWMb (*broken line*) at $T=20$ K. The bands have been obtained from the experimental spectra by subtracting the contributions due to the N band; they have also been suitably shifted along the frequency axis and normalized. Note the stronger coupling of SWMb with high frequency modes

broadening. This is due to the fact that at high temperatures an increase of σ^2 values much larger than that predicted by (2) is observed. A proper fitting of (2) and (3) to the experimental data of both σ^2 and ν_0 can be performed in the range 20–160 K. The continuous lines in Fig. 3 represent such fittings; parameters values are reported in Table 2.

Data in Table 2 show that, for Hb and SWMb, coupling with low frequency modes is at least as relevant as for the CO derivatives of the same hemeproteins and that values of NS and $\langle\nu\rangle$ are remarkably similar. This fact indicates that motions involving the entire heme pocket (and not only CO librations as suggested by Srajer and Champion 1991) contribute to the thermal bath that is coupled to the Soret transition of hemoglobin and myoglobin.

From Fig. 3 it is also evident that deviations from the behavior predicted by (2) and (3) are observed at high temperatures, the effect being analogous to what has been observed for SWMbCO and HbCO. Since (2) and (3) have been derived within the harmonic approximation, we attribute these deviations to the onset of large amplitude non-harmonic nuclear motions. An increase of average atomic fluctuations well above the prediction of the harmonic behavior and occurring at temperatures higher than ≈ 180 K has been observed with a variety of techniques on several biomolecules (Doster et al. 1989; Parak et al. 1981, 1982) and has been attributed to a transition in protein mobility from the low temperature harmonic behavior to a high temperature "liquid like" behavior, likely involving "jumping" among different conformational substates of the biomolecule.

A somewhat different explanation of the deviations from the harmonic behavior evident in Fig. 3 could involve a decrease (at temperature higher than 180 K) of the mean effective frequency ($\langle\nu\rangle$) of the soft modes paralleled by a concomitant decrease of their quadratic coupling

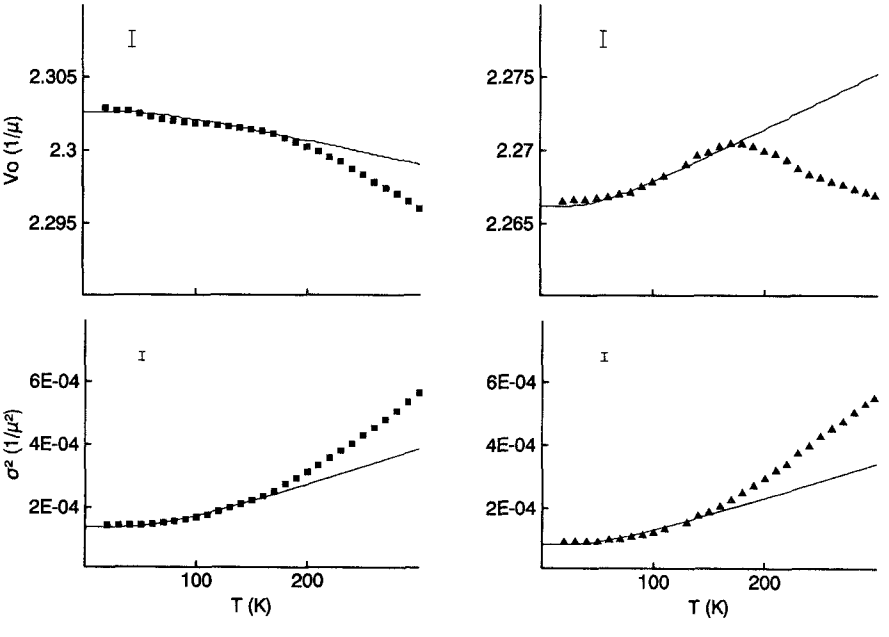

Fig. 3. Temperature dependence of ν_0 (*upper panels*) and σ^2 (*lower panels*) values relative to Hb (*squares, left panels*) and SWMb (*triangles, right panels*). The continuous lines represent fittings of the data in the temperature range 20–160 K in terms of (2) and (3). Typical error bars are shown

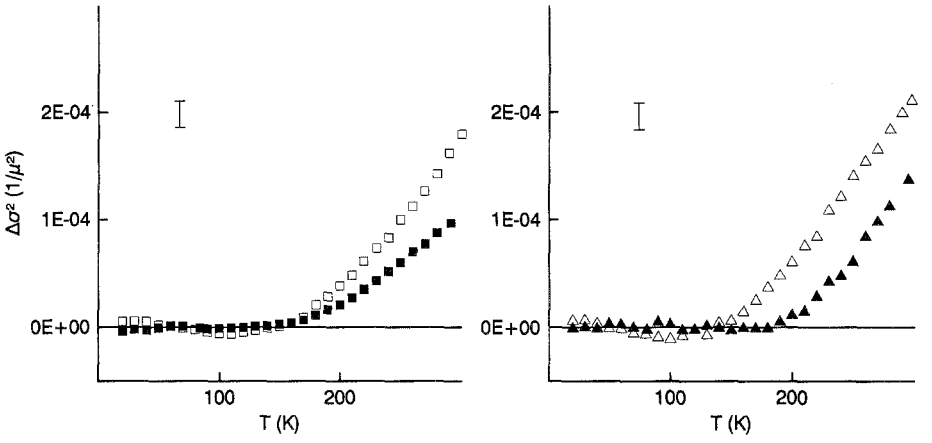

Fig. 4. Differences between σ^2 values and extrapolated harmonic behavior. *Left, squares*: Hb; *Right, triangles*: SWMb. *Full symbols* refer to the CO derivatives and have been taken from Di Pace et al. (1992). Typical error bars are shown

constant (R). Indeed, a $\langle \nu \rangle$ decrease would cause the onset of larger amplitude motions and therefore a σ^2 increase above the predictions of (2); moreover an R decrease would cause a peak frequency red-shift and would explain both the increased red-shift observed for Hb ($R<1$ at low temperatures) and the non-monotonous temperature dependence observed for SWMb ($R>1$ at low temperatures). These anharmonic contributions ($\Delta\sigma^2$, i.e. the difference between the experimental points and the continuous lines in Fig. 3) are reported in Fig. 4 and compared with the analogous quantities for the CO derivatives. From Fig. 4 we see that $\Delta\sigma^2$ values are larger for the deoxy derivatives than for the liganded ones. Data in Fig. 4 point out the influence of the tertiary and quaternary structure on the dynamic properties of hemeproteins and could be attributed to a stabilizing effect of the ligand on the distal heme pocket.

Values of the parameters $Q_0\sqrt{b}$ and $\delta\sqrt{b}$ are shown in Fig. 5 as a function of temperature. From Fig. 5 we note the following points:

i) For SWMb at $T=300$ K the value of $Q_0\sqrt{b}$ is $0.12\,\mu^{-1/2}$. If we assume that the coordinate Q_0 reflects essentially the displacement of the iron atom out of the mean heme plane and if we take from the X-ray diffraction data (Perutz et al. 1987) the value of $Q_0=0.45$ Å, we obtain a value of $b=710\,\text{cm}^{-1}/\text{Å}^2$. A larger value of $b\sim 2\,500\,\text{cm}^{-1}/\text{Å}^2$ has been reported previously by Champion and coworkers (Srajer et al. 1986). From the value of $Q_0\sqrt{b}=0.15$ at $T=300$ K relative to hemoglobin we obtain a Q_0 value of 0.56 Å. This value can be compared with the X-ray data that give, in deoxyhemoglobin, a mean Fe-heme plane distance of 0.58 Å for α chains and of 0.50 Å for β chains (Perutz et al. 1987). We think that the excellent agreement gives strong support to the validity of our spectral analysis.

ii) Both for Hb and SWMb $Q_0\sqrt{b}$ values increase on decreasing the temperature. This indicates that the distance of the iron atom with respect to the heme plane increases on lowering the temperature. In particular, going from 300 to 20 K Q_0 values increase from 0.45 to 0.62 Å for SWMb and from 0.56 to 0.67 Å for Hb. To our knowledge this is one of the first demonstrations of the temperature dependence of the iron-heme plane geometry. The recent finding by Frauenfelder and coworkers (Steinbach et al. 1991) that a temperature dependent distribution of activation enthalpies is needed to explain the rebinding kinetics of CO to Mb after flash photolysis is not in conflict with the present results. The temperature dependence of the iron porphyrin distance had been hy-

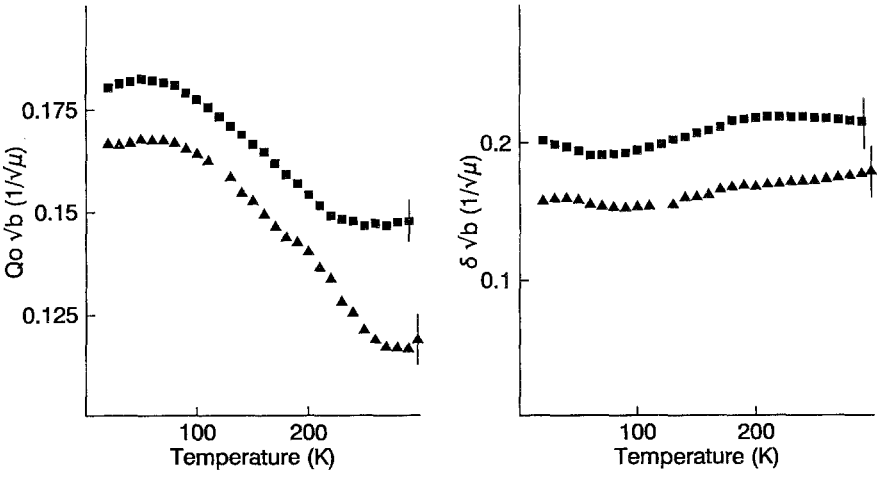

Fig. 5. Temperature dependence of $Q_0\sqrt{b}$ (*left*) and of $\delta\sqrt{b}$ (*right*). *Squares* refer to Hb, triangles to SWMb. Error bars at high temperature are shown; at low temperature the errors decrease by more than a factor of two

pothesized by our group in previous publications (Cordone et al. 1986; Cupane et al. 1988) to explain the increase (upon lowering the temperature) of the integrated intensities of the iron porphyrin charge transfer bands in Hb and SWMb; this hypothesis is confirmed by the present data.

iii) $\delta\sqrt{b}$ values are, both for SWMb and Hb, of the same order of $Q_0\sqrt{b}$ and almost temperature independent. X-ray data at 300 K give $\sqrt{\langle \Delta x^2\rangle_{Fe}} \sim 0.26$ Å, i.e. about a factor 2 smaller than the mean Fe-heme plane distance (0.45 Å). It should be remembered, however, that our data refer to a 65% v/v glycerol/water solution and not to a crystal; moreover, as suggested by Srajer and Champion (1991), other angular coordinates (like the tilting of the Fe-N_{His} bond with respect to the heme normal or the azimuthal orientation of the proximal histidine) can contribute to the overall heme disorder and therefore to the parameter δ. The fact that δ is almost temperature independent argues for its provenance from static disorder and contributes further evidence for the existence of conformational substates in hemeproteins. The larger δ values observed for Hb with respect to SWMb are consistent with the presence of α-β heterogeneity in deoxyhemoglobin.

A comparison of deoxy and CO derivatives indicates that the former appear to be much more heterogeneous (at least from a spectral point of view) than the latter; the idea of a possible functional relevance of conformational heterogeneity may therefore be tempting.

Acknowledgements. We are indebted to Prof. L. Cordone for many useful suggestions and discussions and for a critical reading of the manuscript and to Dr. A. Di Pace for her initial contribution to data analysis. The technical help of Mr. G. Lapis and Mr. M. Quartararo is also gratefully acknowledged. This work has been supported by grants from MURST. General indirect support from CRRNSM is also acknowledged.

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