Protein dynamics

Vibrational coupling, spectral broadening mechanisms, and anharmonicity effects in carbonmonoxy heme proteins studied by the temperature dependence of the Soret band lineshape

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ABSTRACT In this work we study the temperature dependence of the Soret band lineshape of the carbonmonoxy derivatives of sperm whale myoglobin, human hemoglobin,and its isolated α and β subunits. To fit the observed spectral profile we use an analytic expression derived for a system whereby a single electronic transition is coupled to Franck-Condon active vibrational modes, within the adiabatic and harmonic approximation. The vibronic structure of the spectra arises from the coupling with high frequency modes; these modes contribute to the total line shape through a series of Lorentzians with peak positions at vibrational overtones and half width related to the time constant of the population decay of the excited electronic state (homogeneous broadening); moreover, the coupling with low frequency modes broadens each Lorentzian to a Voigtian. Inhomogeneous broadening is modeled as a gaussian distribution of the 0–0 transition frequencies and is therefore added as a constant term to the previous gaussian width.

This spectral deconvolution enables us to investigate the different contributions to line broadening and the parameters that characterize the vibrational coupling, as well as their dependence upon protein and solvent composition. The investigation is carried out as a function of temperature in the range 20–300 K; relevant information is obtained by comparing experimental results with theoretical predictions.

This work supports a description of the investigated proteins as heterogeneous systems, whose heterogeneity depends on the particular protein and on the composition of the external matrix.

The delocalized π electron cloud of the porphyrin ring is coupled not only to the high frequency vibrational modes of the active site but also to a "bath" of lower frequency modes that involve the entire protein; moreover at suitable temperatures ($\approx$200 K), anharmonic motions, which are an obvious prerequisite for the jumping among different conformational substates, become evident.

INTRODUCTION

The optical absorption spectra of hemeproteins exhibit an intense band at ~400 nm (Soret band) whose molar extinction coefficient is larger than 100,000 M^{-1} cm^{-1}; this band is attributed to an electric dipole allowed $\pi \rightarrow \pi^*$ transition of the delocalized electron cloud of the heme group (Eaton et al., 1978; Eaton and Hofrichter, 1981; Makinen and Churg, 1983). Due to the coupling between the electronic transition and the motion of the nearby nuclei, the band narrows and its peak frequency shifts as the temperature is lowered. The temperature dependence of the optical spectra of various metallo proteins has been extensively studied by our group in recent years (Cordone et al., 1986, 1988; Cupane et al., 1988, 1990a,b; Leone et al., 1987). In particular, the Soret band of various ferrous derivatives (both liganded and unliganded) of hemoglobin (Cordone et al., 1986) and myoglobin (Cupane et al., 1988) has been heuristically deconvoluted in gaussian components; the first (M_1) and second (M_2) moments of the whole band have been obtained and their thermal behavior has provided some information on the dynamic properties of these proteins in the proximity of the chromophore. However, the lack of a suitable theoretical treatment for the thermal evolution of the band profile did not enable us to extract from the data information on the electron-vibration coupling constants and on the various spectral broadening mechanisms that contribute to the Soret band linewidth.

Given the large extinction coefficient, the interaction of the electronic transition responsible for the Soret band with nuclear vibrations can be fairly well described within the Franck-Condon approximation and higher order effects (e.g., Herzberg-Teller vibronic coupling [Spiro, 1983]) can be neglected; moreover, the resonance Raman spectra (Soret excitation) of these hemeproteins are well known (Spiro, 1983; Tsubaki et al., 1982; Asher, 1981; Bangcharoenpaurpong et al., 1984). These facts have stimulated us to develop an analytical expression that is apt to give a non-heuristic deconvolution of the measured Soret spectra at various temperatures. To this purpose, we consider a system in which a single electronic transition is coupled to Franck-Condon active vibrational modes within the adiabatic and harmonic approximations. Making use of the so-called "short times approximation" (Chan and Page, 1983), the spectra consist of a sum of Voigtians (Gaussian convolutions of Lorentzians) in which the Lorentzian linewidth takes into account the homogeneous broadening due to the nonradiative population decay of the excited electronic state, and the Gaussian linewidth takes into account both the coupling with a "bath" of low frequency modes and the presence of inhomogeneous broadening. We use this approach to study the Soret band of the carbonmonoxy derivatives of human hemoglobin (HbCO), sperm whale myoglobin (SW-MbCO), and isolated α and β subunits of human hemoglobin (αCO and βCO, respectively). The same approach has been exploited by Champion and co-workers to study the Soret Band of SW-MbCO (Srajer et al., 1986) and of

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cytochrome *c* (Schomacker and Champion, 1986), using the frequencies and coupling constants of high frequency modes obtained from Raman spectroscopy. In the case of SW-MbCO, however, only the spectra at $T = 10$ and $T = 290$ K were analyzed. The analysis of the full temperature dependence in the range 300–20 K reported in this paper enables to obtain more complete information on the various contributions to line broadening and to put in evidence the onset, at temperatures higher than $\approx$200 K, of anharmonic motions. Moreover, the extension of the study to two different solvent compositions gave information also on the dependence of the dynamic properties studied upon the structure of the surrounding matrix.

THEORETICAL SECTION

To obtain an analytical expression for the band profile at various temperatures we consider a single electronic transition coupled with N harmonic, Franck-Condon active, vibrational modes. In our case, these are the normal modes of the system heme + ligand + proximal histidine that in view of the close-packed structure of heme pocket are modulated by low frequency modes of the protein moiety.

We treat our system within the Born-Oppenheimer and Condon approximations, i.e., we describe a state with a factorized wave function $\Psi(r, X) = \varphi(r)\chi(X)$, where $\varphi(r)$ is a purely electronic wavefunction and $\chi(X)$ is the nuclear wavefunction that depends parametrically upon the electronic state but not on the electronic coordinate. In the approximation of electron-nuclei linear coupling the energy difference between the excited and ground state can be written as:

$$\bar{\nu} = \nu_{00} + \sum_j^N \nu_j(n'_j + 1/2) - \sum_j^N \nu_j(n_j + 1/2), \qquad (1)$$

where ν_{00} is the frequency of the purely electronic (0–0) transition, ν_j is the frequency of the jth normal mode, n_j and n'_j are the occupation numbers of the jth normal mode in the ground and excited state, respectively.

The optical absorption spectrum is given by (Dexter, 1958):

$$A(\nu) = M\nu \sum_{\{n_j, n'_j\}} P(\{n_j\})|\langle\chi'|\chi\rangle|^2 \frac{\Gamma}{(\nu - \bar{\nu})^2 + \Gamma^2}, \qquad (2)$$

where M is a constant proportional to the square of the electric dipole moment; the parameter Γ is a damping factor (homogeneous broadening) related to the finite lifetime of the excited state (in the case of the Soret band of hemeproteins, Γ is thought to arise only from nonradiative processes, because linewidth contributions related to pure electronic dephasing are negligible [Champion and Lange, 1980; Champion and Perreault, 1981]); $P(\{n_j\})$ is the probability of having the ground state with a set of nuclear occupation numbers $\{n_j\}$, i.e., with an energy $\Sigma\nu_j(n_j + 1/2)$, in particular $P(\{n_j\}) = \Pi p(n_j)$ with

$$p(n_j) = \frac{e^{-n_j(h\nu_j/K_BT)}}{\sum_n^\infty e^{-n(h\nu_j/K_BT)}} = 2e^{-(n_j+1/2)(h\nu_j/K_BT)} \sinh \frac{h\nu_j}{2K_BT};$$

χ and χ' are the nuclear wave functions relative to the ground and excited state respectively:

$$\chi = \prod_j^N \chi_j(q_j) \quad \chi' = \prod_j^N \chi_j(q_j - \Delta_j),$$

where χ_j is the generic eigenstate of a harmonic oscillator with frequency ν_j, q_j is the nuclear normal coordinate, Δ_j is the displacement of nuclear equilibrium positions between the excited and ground state.

Following Chan and Page (1983), we separate the entire set $\{N\}$ of normal modes into a subset $\{N_h\}$ of high frequency modes ($h\nu_j \gg K_BT$, so that $\langle n_j\rangle = 0$) and a subset $\{N_l\}$ of low frequency "soft" modes (ν_j smaller than the observed bandwidth). The coupling with high frequency modes brings about effects not dependent upon temperature and is responsible for the vibronic structure of the band profile. The coupling with the soft modes contributes to the overall spectral linewidth with a Gaussian broadening term that is temperature dependent. In fact, by using the so-called "short times approximation", Eq. 2 can be reduced to a sum of Voigtian functions (Gaussian convolutions of Lorentzians) whereby the temperature dependence of the spectrum arises only from the Gaussian broadening term. To take into account eventual shifts of the peak positions with temperature, quadratic coupling must also be considered. In this case, not only the nuclear equilibrium positions but also the vibrational frequencies depend upon the electronic state. It is possible to take into account the effects of quadratic coupling by substituting in Eq. 1, the parameter ν_{00}, with

$$\nu'_{00} = \nu_{00} - \frac{1}{4}\sum_j^N \frac{\nu_j^2(g) - \nu_j^2(u)}{\nu_j(g)}(2\langle n_j\rangle + 1),$$

where $\nu_j(g)$ and $\nu_j(u)$ are the frequency of the jth normal mode when the electron is in the ground or excited state, respectively, and

$$\langle n_j\rangle = \frac{1}{e^{h\nu_j/K_BT} - 1}$$

is the occupation number of the jth normal mode.
Eq. 2 can be expressed in the form:

$$A(\nu) = M\nu \sum_{m_1,\ldots,m_{N_h}}^\infty \prod_i^{N_h} \frac{S_i^{m_i} e^{-S_i}}{m_i!} \times \frac{\Gamma}{\left[\nu - \nu_0 - \sum_i^{N_k} m_i R_i \nu_i(g)\right]^2 + \Gamma^2} \otimes \frac{1}{\sigma} e^{-\nu^2/2\sigma^2}, \qquad (3)$$

where the symbol $\otimes$ indicates the convolution operator, $R_j = \nu_j^2(u)/\nu_j^2(g)$ is the quadratic coupling constant,

$$S_j = \frac{(2\pi)^2 \nu_j}{h} \frac{\Delta_j^2}{2}$$

is the linear coupling constant of the electronic transition with the jth normal mode,

$$\sigma^2 = \sum_k^{N_l} S_k R_k^2 \nu_k^2(g) \coth \frac{h\nu_k(g)}{2K_B T} \quad (4)$$

is the gaussian width, and

$$\nu_0 = \nu_{00} - \frac{1}{4} \sum_j^N \frac{\nu_j^2(g) - \nu_j^2(u)}{\nu_j(g)} \times \coth \frac{h\nu_j(g)}{2K_B T} + \sum_k^{N_l} S_k R_k \nu_k(g). \quad (5)$$

It can be seen that the spectrum results from the superposition of a series of Voigtians (one for each high frequency mode); within each series the Voigtians are spaced by quantities multiple of the frequency of the vibrational mode. As the temperature is lowered, the peak frequencies of all the Voigtians shift and the width of the Gaussian term narrows; these temperature effects are related to the coupling with low frequency modes.

By considering the weak coupling with a "bath" of soft modes, Eqs. 4 and 5 can be rewritten as:

$$\sigma^2 = S_l R_l^2 \nu_l^2 \coth \frac{h\nu_l}{2K_B T} \quad (6)$$

$$\nu_0 = \nu_{00} - \frac{1}{4} \nu_l (1 - R_l) \coth \frac{h\nu_l}{2K_B T} + C, \quad (7)$$

where ν_l, S_l and R_l are the effective frequency, linear, and quadratic coupling constants of the low frequency bath, whereas C takes into account temperature independent contributions arising from linear coupling with low frequency modes and quadratic coupling with high frequency modes.

If conformational heterogeneity is present, one has to perform averages over all spectrally different species. As a consequence, the parameters appearing in Eqs. 3–7 become average effective values and a further contribution to the overall linewidth (inhomogeneous broadening) appears. Following an approach widely used in the literature (Schomacker and Champion, 1986), we model this effect as a Gaussian distribution of 0–0 frequencies; inhomogeneous broadening, therefore, contributes a further term to the width of the Gaussian component of the Voigtian. Eq. 6 can therefore be rewritten as:

$$\sigma^2 = S_l R_l^2 \nu_l^2 \coth \frac{h\nu_l}{2K_B T} + \sigma_{in}^2. \quad (8)$$

If the conformational substates of the protein are not fully degenerate, the σ_{in}^2 increase with temperature is expected at temperatures whereby interconversion between substates takes place.

MATERIALS AND METHODS

Samples

Sperm whale myoglobin (SW-Mb) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Appropriate amounts of lyophilized ferric proteins were dissolved in 0.1 M phosphate buffer, pH 7, to give protein solutions of $\sim 6 \times 10^{-4}$ M in heme; these solutions were centrifuged, equilibrated with CO, and reduced by the anaerobic addition of $\sim 3 \times 10^{-2}$ M sodium dithionite.

Human hemoglobin (Hb) was prepared from the blood of a single donor, as already described (Cordone et al., 1979).

α and β subunits from human hemoglobin were a kind gift of Dr. E. E. Di Iorio (Eidgenössische Technische Hochschule, Zürich) and were prepared as already reported (Di Iorio et al., 1991).

All samples for spectrophotometric measurements were prepared by diluting the concentrated protein solutions into water-cosolvent-buffer mixtures previously equilibrated with CO and containing 65% vol/vol glycerol or ethylene glycol in water, 0.1 M phosphate buffer (pH 7, in water at room temperature), and $\sim 3 \times 10^{-4}$ M sodium dithionite. Final protein concentrations were 6×10^{-6} M (in heme).

Glycerol (Carlo Erba, Milan, Italy) and ethylene glycol (Fluka, Buchs, Switzerland) were analytical grade and were used without further purification.

Instrumentation

Spectra (500–300 nm) were taken with a Jasco Uvidec 650 spectrophotometer (Japan Spectroscopic Co., Tokyo) (bandwidth = 0.4 nm; time constant = 1 s; scan speed = 40 nm/min). The experimental setup and methods for optical measurements in the temperature range 300–20 K have been described previously (Cordone et al., 1986). The baseline (cuvette + solvent + buffer) measured at room temperature was subtracted from each spectrum. In fact, the absorption of dithionite at the concentration used is negligible (sodium dithionite has an absorption band at 314 nm, with an extinction coefficient of 8 mM^{-1} cm^{-1} [Di Iorio, 1981]); moreover, it is known that in the spectral region of interest the baseline does not depend on temperature. We stress that our samples remained homogeneous and transparent at all temperatures and that cracks were not observed.

Data analysis

Spectral data were recorded at 0.8-nm intervals and deconvolution in terms of Eq. 3 was performed on a HP-1000 computer (Palo Alto, CA); the mean square deviation was minimized using a nonlinear least-squares algorithm. Errors on fitted parameters are 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 500 nm (20,000 cm^{-1}) has been brought to zero for all the spectra; moreover, a Gaussian component centered at $\sim$29,000 cm^{-1} was added to Eq. 3 to take into account contributions due to the N band.

Fittings are performed in terms of Eq. 3. M, Γ, S_i, σ, ν_0 are fitting parameters. ν_i values, relative to high frequency modes, are known from resonance Raman (RR) spectra reported in the literature (Tsubaki et al., 1982; Asher, 1981; Bangcharoenpaurpong et al., 1984) (using Eq. 3 we have assumed $R_i \nu_i \sim \nu_i$, indeed, by analyzing the ν_0 temperature dependence, R_i values in the range 0.99–1.01 are obtained; see Table 3); only the most coupled modes, corresponding to ν_1 = 350 cm^{-1}, ν_2 = 676 cm^{-1}, ν_3 = 1,100 cm^{-1}, ν_4 = 1,374 cm^{-1}, are taken into account because the other high frequency, less coupled modes do not significantly contribute to the observed band profile. 676 and 1374 cm^{-1} modes correspond to very sharp lines in RR spectra. We note that 350 and 1,100 cm^{-1} are "average effective" frequencies accounting for two spectral regions characterized by several, quasi degen-

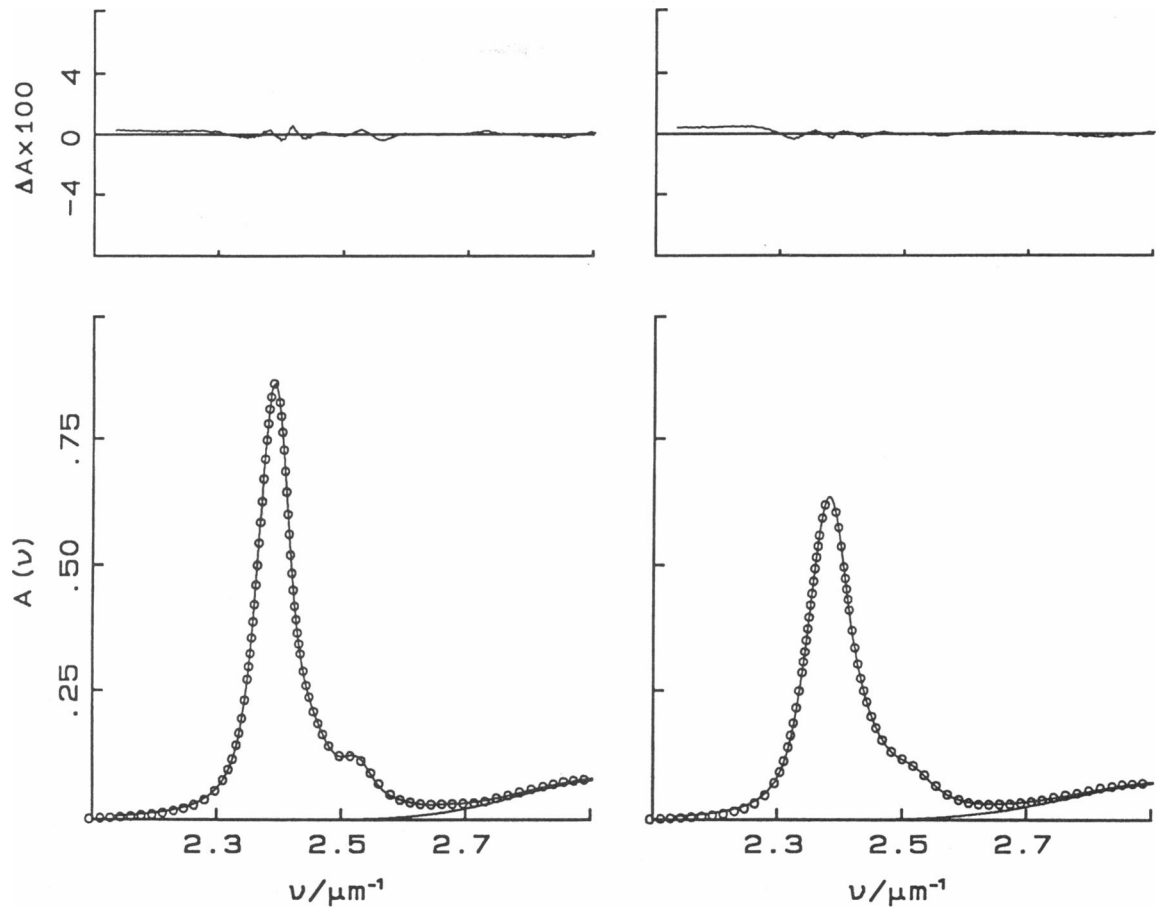

FIGURE 1 Spectra of hemoglobin in 65% glycerol/water at T = 20 K (*left*) and T = 291.5 K (*right*). Circles are the experimental points; the continuous lines represent fittings in terms of Eq. 3. For the sake of clarity not all the experimental points are included. The residuals are also reported in the upper panels, on an expanded scale.

erate, peaks. We also tried fittings whereby the coupling with the Fe-CO stretching mode at ~510 cm^{-1} was also considered; because results indicated very negligible coupling, this mode has been discarded in our analysis. Concerning the 350-cm^{-1} mode, its assignment to the set of high frequency modes ($h\nu \gg K_BT$) is quite a good approximation at low temperature and a less good one at room temperature (K_BT/h = 215 cm^{-1} at T = 300 K). We treat the 350-cm^{-1} mode as a high frequency one; in order to reduce fitting ambiguities arising from broadening of the band and consequent lack of a clearly resolved vibronic structure at high temperatures, we fixed the parameter S_{350} to its low temperature value. Ambiguities due to low resolution are also found for the parameter S_{1100} that has been fixed to its low temperature value too.

In the fitting procedure the Voigtians appearing in Eq. 3 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 Γ and σ (the greater σ/Γ, the slower the convergence). In our cases the ratio σ/Γ is never less than ~½ and the convergence is ensured by truncating the fraction at about the 100th step.

We have also studied the convergence of the infinite sum in Eq. 3. For large m_i values, the term $S^{mi}e^{-Si}/m_i!$ 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 to truncate the sum at m_i never greater than 2.

RESULTS AND DISCUSSION

Fig. 1 shows typical spectra and fittings at low and high temperature, together with the residuals, on an expanded scale.

Table 1 shows the low temperature values of the linear coupling constants of the high frequency modes for the

TABLE 1 **Low temperature linear coupling constants and Γ values for various hemeproteins in 65% ethylene glycol/water**

	S_{350}	S_{676}	S_{1100}	S_{1374}	Γ
					cm^{-1}
α	0.026 ± 0.013	0.071 ± 0.008	0.014 ± 0.007	0.078 ± 0.006	239 ± 4
β	0.068 ± 0.015	0.089 ± 0.009	0.025 ± 0.008	0.083 ± 0.007	209 ± 7
Hb	0.047 ± 0.009	0.073 ± 0.005	0.016 ± 0.005	0.079 ± 0.004	215 ± 4
SW-Mb	0.116 ± 0.017	0.056 ± 0.010	0.018 ± 0.009	0.092 ± 0.008	211 ± 7

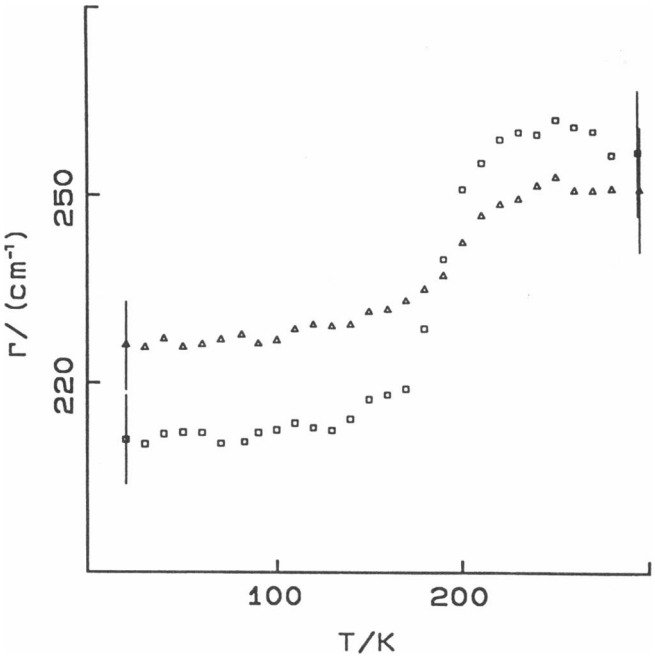

FIGURE 2 Temperature dependence of the homogeneous width Γ for myoglobin. 65% glycerol/water (△). 65% ethylene glycol/water (□). Error bars are shown for high and low temperature points.

various proteins in 65% ethylene glycol/water; effects on S values due to variations of solvent composition were always within the experimental errors; moreover, the S values are found temperature independent, in agreement with previous reports in the literature (Schomacker and Champion, 1989), although a slight but systematic (~20%) increase of S_{676} at high temperatures was observed for all the proteins investigated. Concerning the S values relative to the modes at 676 and 1374 cm^{-1} (that correspond to sharp peaks in the RR spectra of these hemoproteins), we stress that their values agree rather well with those from the Raman excitation profiles estimated by Bangcharoenpaurpong et al. (1984) for the deoxy derivatives; this agreement gives support to the validity of our spectral deconvolution procedure and to the significance of the parameter values reported in Table 1. From Table 1 we note that, for all the modes considered, S values relative to hemoglobin are larger than those of α chains and smaller than those of β chains, while myoglobin appears not systematically correlated to isolated chains.

The low temperature values of the homogeneous linewidth (Γ) are also reported in Table 2: they are of the order of 200–250 cm^{-1}, in agreement with previous reports in the literature; in particular for SW-MbCO, the values of $\Gamma = 211 \pm 7$ cm^{-1} reported in this work compare very well with the value of 217 cm^{-1} reported by Srajer et al. (1986). For hemoglobin and its isolated subunits, moreover, Γ values are not significantly affected by solvent composition and seem not to depend on temperature also at high temperature. We think that the unambiguous Γ determination reported in this paper is due to the almost Lorentzian shape of the red edge of the band; in fact, the coupling of the transition with nuclear oscillation barely affect the shape of the band red edge. Myoglobin, again, behaves in a different way: in fact, as reported in Fig. 2, Γ values depend upon temperature and solvent composition and undergo an abrupt upward shift at temperatures whereby the solvents approach glass transition (Cordone et al., 1988), i.e., ≈160 K for the sample in 65% ethylene glycol and ≈180 K for the sample in 65% glycerol. To check that effects reported in Fig. 2 are not due to fitting artifacts we have also performed fittings of the high temperature spectra in 65% vol/vol ethylene glycol, whereby the parameter Γ was fixed to its low temperature value. In such fittings χ^2 values increased by a factor of ~1.8, whereas no sizeable variations in the values of the other parameters (except for a ≈10% σ increase) were observed. As already mentioned in the Theoretical Section, the parameter Γ is related to the time constant of the nonradiative energy decay from the excited state that, in turn, is determined by the dependence of the electronic wave function upon nuclear coordinates (non-Born-Oppenheimer effects). Our data indicate that in the case of CO-myoglobin these properties are influenced by the composition and physical state (glass vs. liquid) of the external matrix. The abrupt Γ decrease observed by lowering the temperature closely parallels the behavior of the peak frequency of the visible and Soret bands (see references 6–8 and Fig. 6d) and could be related to an heme pocket readjustment that brings about, e.g., a tilting of the bound CO molecule towards the heme normal (Di Iorio et al., 1991).

The σ^2 temperature dependence is reported in Fig. 3. It is evident that Eq. 8 cannot be fit to σ^2 values in the whole temperature range 20–300 K; indeed, Eq. 8 requires, in the limit $K_BT \gg h\nu_l/2$, the σ^2 thermal behavior to be given by a straight line whose intercept at $T = 0$ K is σ^2_{in}. From Fig. 3, it is evident that a straight line tangent to the high temperatures experimental points would have a negative intercept at $T = 0$ K, and in turn this would clearly imply unphysical negative σ^2_{in} values. This is due to the fact that at high temperatures an increase of σ^2 values much larger than that predicted by Eq. 8 is observed. A proper fitting of Eq. 8 to the experimental data can be performed in the range 20–160 K; this, however, due to the limited temperature range and number of data points, gives parameters that, although physical, are barely determined. To overcome this difficulty we proceeded as follows: because (see Fig. 3) the effect of solvent composition on σ^2 consists in an upward parallel shift of the σ^2 values in 65% glycerol with respect to those in 65% ethylene glycol, we performed for each protein fittings of the data in both solvents using the same values for parameters S and ν_l and different values for the parameter σ_{in}. The continuous lines reported in Fig. 3 represent such fittings; the values of the parameters are re-

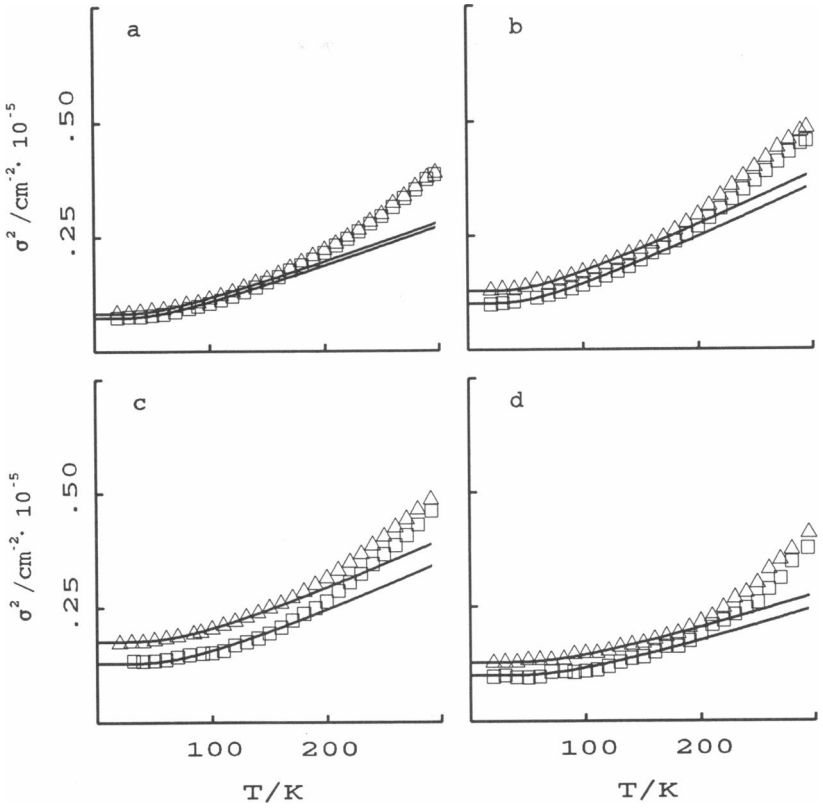

FIGURE 3 Temperature dependence of σ^2 values for (a) α chains, (b) β chains, (c) hemoglobin, (d) myoglobin. 65% glycerol/water (△). 65% ethylene glycol/water (□). The continuous lines represent fittings of the data in the temperature range 20–160 K in terms of Eq. 8.

ported in Table 2; although their standard deviations remain rather large, some qualitative conclusions can be drawn.

(a) The coupling with a bath of low frequency modes (soft modes) is relevant, with values of the linear coupling constants in the range 0.3–0.7; moreover S values relative to isolated hemoglobin chains are larger than those relative to hemoglobin and to myoglobin. We remind that, in our definition (see Theoretical Section), "soft modes" are those whose occupation number is substantially different from zero in the temperature range investigated ($K_BT/h \approx 215$ cm^{-1} at room temperature) and whose frequency is smaller than the observed bandwidth.

TABLE 2 Values of the parameters obtained by fitting the low temperature σ^2 behavior in terms of Eq. 8

	S	ν_1	σ_{in}
		cm^{-1}	cm^{-1}
α 65% et-gly	0.52 ± 0.2	120 ± 10	<30
α 65% glyc			30 ± 23
β 65% et-gly	0.65 ± 0.2	125 ± 10	<40
β 65% glyc			52 ± 17
Hb 65% et-gly	0.47 ± 0.2	170 ± 20	<55
Hb 65% glyc			70 ± 23
SW-Mb 65% et-gly	0.29 ± 0.2	180 ± 30	<50
SW-Mb 65% glyc			53 ± 38

Standard deviations are obtained from the fittings by inversion of the curvature matrix; when uncertainties in the parameters values are too large we prefer to give only estimates of upper limits.

(b) The mean effective frequencies of the soft modes range from 120 to 180 cm^{-1}, the isolated hemoglobin subunits having characteristic frequencies lower than hemoglobin or myoglobin; this fact and the smaller S value observed for hemoglobin could be explained by considering that a tetrameric structure must have force constants higher than its constituent subunits. Isolated α and β chains have been reported to be more flexible than Mb (Di Iorio et al., 1991); the effective frequencies and S values of the soft modes reported in this paper confirm this suggestion. In the case of SW-MbCO, Srajer et al., (1986) reported a $\nu_1 S_1$ value of 120 cm^{-1}. From our analysis in the temperature range 20–160 K we obtain ν_1 = 180 cm^{-1} and S_1 = 0.3. In our opinion the discrepancy arises from the fact that Srajer et al., (1986) derive their values from the analysis of the T = 10 and T = 290 K spectra and not from the entire σ^2 temperature dependence. In fact, if their method is applied using the 20 and 290-K data points we report, the values obtained for $\nu_1 S_1$ and ν_1 are 90 and 100 cm^{-1}, respectively; these values,

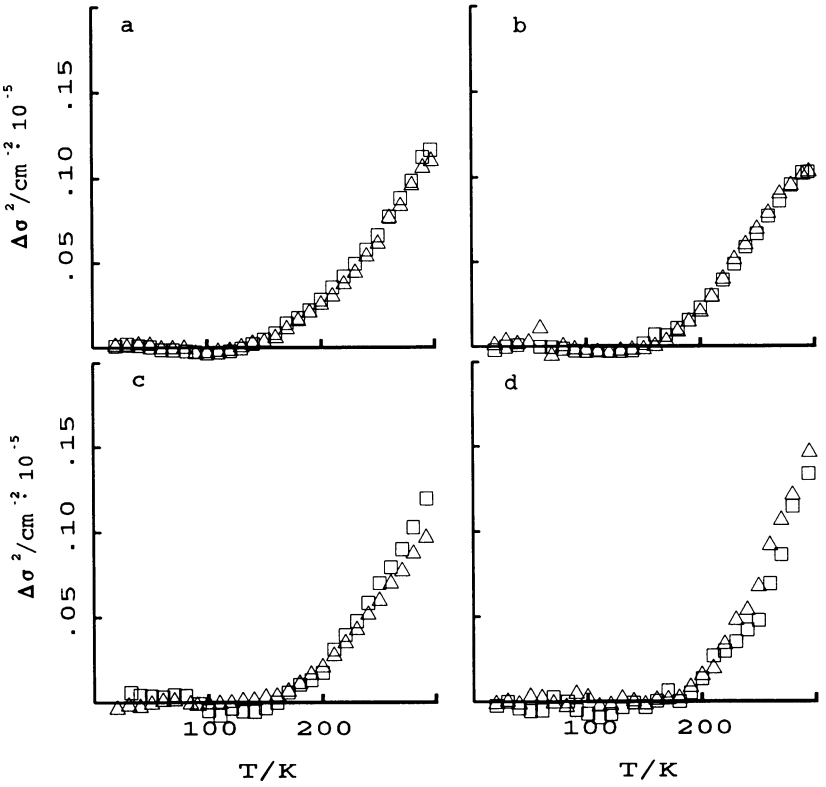

FIGURE 4 Differences between σ^2 values and extrapolated harmonic behavior (*a*) α chains, (*b*) β chains, (*c*) hemoglobin, (*d*) myoglobin. 65% glycerol/water (△). 65% ethylene glycol/water (□).

however, if used in Eq. 8 do not fit the σ^2 behavior in the whole temperature range.

(*c*) σ_{in} values depend both on the protein and on solvent composition. In particular, α chains appear to be the most homogeneous proteins ($\sigma_{in} \approx 0$) and are almost solvent independent. For the other proteins, the present data show that spectral heterogeneity is smaller in 65% ethylene glycol/water than in 65% glycerol/water. Two previous estimates of the inhomogeneous width of the Soret band of MbCO in 75% glycerol/water are available from the literature and have been obtained by spectral (41 cm^{-1}) and kinetic (13 cm^{-1}) hole burning (Ormos et al., 1990). The presently reported value of 53 ± 38 cm^{-1} agrees well with the above values; moreover, this work also extends to other hemeproteins the measurements of inhomogeneous contributions to the Soret band linewidth. The data clearly indicate that, for all the proteins investigated, inhomogeneous broadening depends upon solvent composition; this fact confirms the suggestion that the distribution of conformational substates of these proteins is influenced by the properties of the external matrix (Ansari et al., 1987). The already reported correlation between spectral and kinetic heterogeneity (Di Iorio et al., 1991) is also confirmed by the present data.

From Fig. 3 it is also evident that for all the proteins investigated, deviations from the σ^2 behavior predicted by Eq. 8 are present at high temperatures; the appearance of these deviations is evidenced in Fig. 4. Since, as reported in the Theoretical Section, Eq. 8 has been derived within the harmonic approximation, we attribute this effect to the onset of nonharmonic nuclear motions coupled to the electronic transition; anharmonicity of nuclear motions is an obvious prerequisite for the jumping among conformational substates of the protein. Data in Fig. 4 seem to depend upon the particular protein and not upon the solvent composition; in particular, for hemoglobin chains and for myoglobin the deviations begin at T = 160 and 190 K, respectively, confirming the suggestion (see point *b* above) that the isolated hemoglobin chains are more flexible than myoglobin. For MbCO, an increase in the average atomic fluctuations of the iron, of backbone and side-chain atoms, and of hydrogen atoms well above the predictions of the harmonic behavior and occurring at temperatures higher than 180 K has been observed both experimentally, by Mössbauer (Parak et al., 1981, 1982) and inelastic neutron scattering (Doster et al., 1989), and by computer simulations (Loncharich and Brooks, 1990; Smith et al., 1990); this effect has been attributed to a transition (occurring at ~200 K) in protein mobility from the low temperature harmonic behavior to a high temperature "liquid-like" behavior involving mainly jumping between conformational substates of the protein.

A comparison of our data with those available in the literature (both from simulation and experiment) is re-

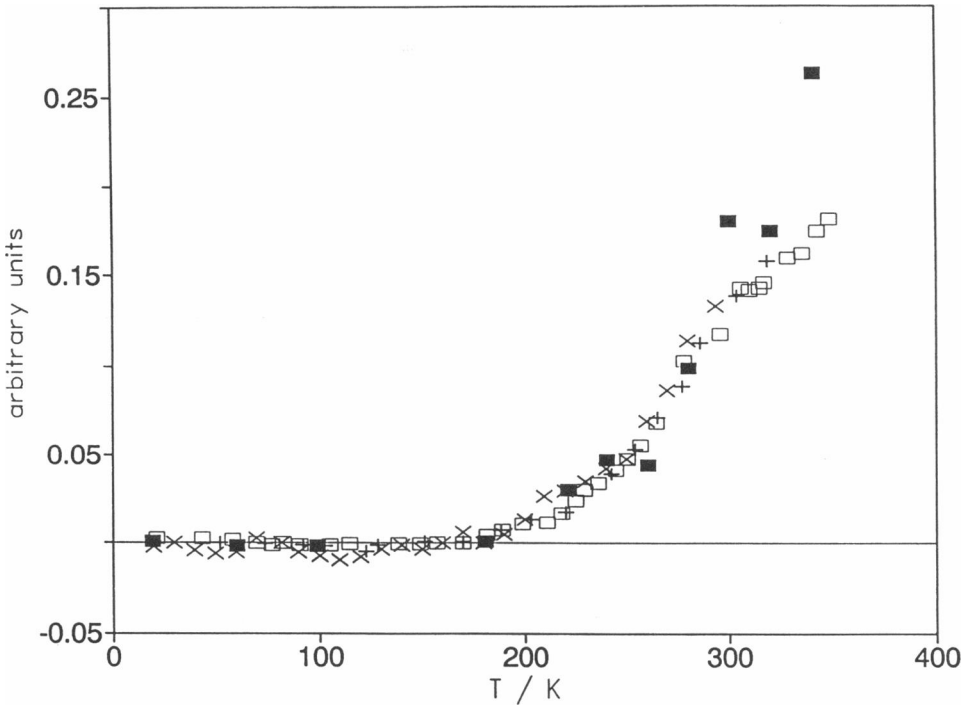

FIGURE 5 Deviations of various quantities from the normal harmonic behavior, as a function of temperature, for myoglobin: (×) $\Delta\sigma^2$ values, present work (see Fig. 4 *d*); (□) $\Delta\langle x^2\rangle$ values relative to the iron atom, obtained from Mössbauer spectroscopy (see Parak et al., 1982); (+) $\Delta\langle x^2\rangle$ values relative to non exchangeable hydrogen atoms of the protein, obtained from inelastic neutron scattering (see Doster et al., 1989); (■) $\Delta\langle x^2\rangle$ values relative to nonexchangeable hydrogen atoms of the protein, obtained from molecular dynamics simulation (see Loncharich and Brooks, 1990). The scales of the ordinates have been suitably normalized.

ported in Fig. 5: the agreement is striking. The fact that different techniques that probe different parts of the molecule (the iron atom, Mössbauer spectroscopy; the delocalized π electron cloud of the porphyrin, optical spectroscopy; the non exchangeable hydrogen atoms, inelastic neutron scattering) all give very similar results implies that the anharmonicity evidenced in Figs. 4 and 5 involves the entire protein and motions occurring over a very wide time scale.

In previous reports on the temperature dependence of the second moment of the Soret band of HbCO and SW-MbCO, such deviations from the behavior predicted by Eq. 8 were not noticed (Cordone et al., 1986, 1988; Cupane et al., 1988; Leone et al., 1987). We recall that the overall bandwidth (M_2) is determined not only by the coupling to the bath of low frequency modes (σ^2) but also by terms arising from homogeneous broadening and from the coupling to the high frequency modes. The addition of these large contributions masks the anharmonic behavior of σ^2 reported in Figs. 3 and 4.

Figure 6 shows the ν_0 temperature dependence for the various proteins investigated. The data in Fig. 6 are in full agreement with the analogous ones relative to the temperature dependence of the first moments (M_1) of the Q_0 and Q_v bands of the same heme proteins reported in a previous publication (Di Iorio et al., 1991). In particular, α chains display the largest low temperature ν_0 value that is barely influenced by solvent composition. For β chains ν_0 values are solvent dependent (the values in ethylene glycol being larger than those in glycerol) and smaller than those relative to α chains; moreover, a sigmoidal behavior, more pronounced in ethylene glycol, is observed. For hemoglobin the ν_0 temperature dependence is intermediate between those of the isolated subunits. For myoglobin, a solvent dependent ν_0 transition, occurring at the temperatures of glass transition of the matrix, is observed. This behavior has been found also in previous investigations (Cupane et al., 1988; Cordone et al., 1988) on the temperature dependence of the first moment (M_1) of the Soret and visible bands of oxy, deoxy and, CO-myoglobin and, also due to its solvent dependence, has been attributed to a conformational transition of the heme pocket in myoglobin, slaved to solvent, that causes an alteration of the parameter ν_{00} in Eq. 7. This effect, evident (although to a lesser extent) also in β chains, introduces a further contribution (solvent effect) in the ν_0 temperature dependence.

The continuous lines in Fig. 6 are the best fits of low temperature points in terms of Eq. 7. In such fittings the values of the parameter ν_1 have been fixed to those found from analogous fittings of the σ^2 temperature dependence. Parameter values are reported in Table 3; as can be seen, the values of the quadratic coupling constants (R) are in the range 0.99–1.01 for all the proteins investi-

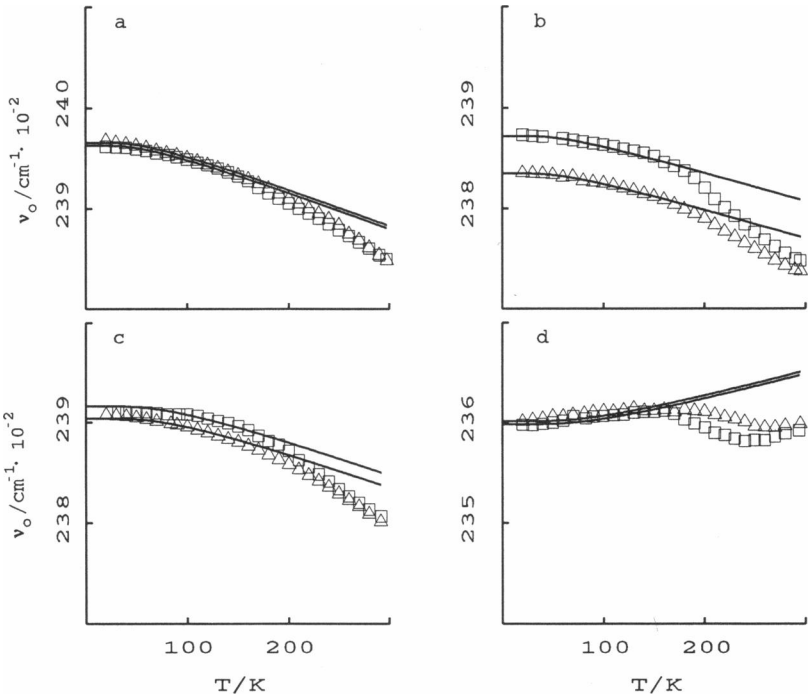

FIGURE 6 ν_0 temperature dependence for (*a*) α chains, (*b*) β chains, (*c*) hemoglobin, (*d*) myoglobin. 65% glycerol/water (△). 65% ethylene glycol/water (□). The continuous lines represent fittings of the data in the temperature range 20–160 K in terms of Eq. 7.

gated. In analogy with the σ^2 behavior, deviations from the "harmonic" behavior are evident at high temperatures.

CONCLUSIONS

The reported results put forward two relevant classes of dynamic properties of the investigated systems. (*a*) Properties not affected by the composition of the solvent matrix are essentially: (*i*) the "high frequency" vibrational modes of the heme group and their coupling constants with the electronic transition investigated; these, as it is to be expected, barely depend upon the particular protein; (*ii*) the "low frequency" vibrational modes, whose mean effective frequency and coupling constants depend upon the particular protein, its tertiary structure, and aggregation state; (*iii*) the anharmonic contributions to nuclear motions that are protein dependent and become evident at high temperatures; anharmonicity of motions is an obvious prerequisite for the jumping among different conformational substates of the protein and therefore for its functional behavior.

(*b*) Properties depending both upon the particular protein and upon composition and physical state of the external matrix are essentially: (*i*) the conformational heterogeneity, as monitored by σ_{in} values (see Table 2), that for all the proteins investigated is larger for samples in glycerol/water than for samples in ethylene glycol/water; (*ii*) the transition observed, for myoglobin, in the Γ and ν_0 temperature dependence and occurring at the temperatures of the glass transition of the matrix; these effects can be attributed to a localized conformational transition in the heme pocket of myoglobin that causes an alteration of the heme-CO geometry that, however, does not affect the vibrational modes nor their coupling constants.

TABLE 3 Values of the parameters obtained by fitting the low temperature ν_0 behavior in terms of Eq. 7

	R_1	ν_1	$\nu_{00} + C$
		cm^{-1}	$cm^{-1}\ 10^{-4}$
α 65% et-gly	0.990 ± 0.001	120	2.3994 ± 0.0001
α 65% glyc			2.3997 ± 0.0001
β 65% et-gly	0.992 ± 0.001	125	2.3898 ± 0.0001
β 65% glyc			2.3861 ± 0.0001
Hb 65% et-gly	0.990 ± 0.006	170	2.3957 ± 0.0003
Hb 65% glyc			2.3944 ± 0.0003
SW-Mb 65% et-gly	1.008 ± 0.002	180	2.3564 ± 0.0004
SW-Mb 65% glyc			2.3567 ± 0.0004

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