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Thermal broadening of the Soret band in heme complexes and in heme-proteins: role of iron dynamics

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Abstract. We report the thermal broadening of the Soret band in heme-CO, heme-OH and protoporphyrin IX in the temperature range 300–20 K. For protoporphyrin IX the temperature dependent Gaussian line broadening follows the behavior predicted by the harmonic approximation in the entire temperature range investigated. In contrast, for heme-CO and heme-OH the harmonic behavior is obeyed only up to about 180 K and an anomalous line broadening increase is observed at higher temperatures. This effect is attributed to the onset of anharmonic motions of the iron atom with respect to the porphyrin plane. Comparison with previously reported analogous data for heme proteins enables us to suggest that the onset of substate interconversions in these latter systems can be reflected in motions of the iron atom with respect to the porphyrin plane.

Key words: Protein dynamics – Optical spectroscopy – Protoporphyrin IX

Introduction

Optical absorption spectra of heme proteins and heme complexes exhibit an intense absorption band at ≈400 nm (Soret band), attributed to a $\pi \rightarrow \pi^*$ electronic transition (Eaton et al. 1978; Eaton and Hofrichter 1981; Makinen and Churg 1983). The interaction of the "optic electrons" with nuclear vibrations can be described within the framework of the Franck-Condon approximation; in particular, the coupling with low frequency modes, whose populations varies within the temperature range investigated, is expected to cause a temperature dependent Gaussian broadening of the absorption line shape. The Gaussian width can be expressed as follows (Di Pace et al. 1992):

$$\sigma^2(T) = N S_1 R_1^2 \nu_1^2 \coth[h\nu_1/2 K_B T] + \sigma_{in}^2 \quad (1)$$

where K_B is the Boltzmann constant, N, ν_1, S_1 and R_1 are respectively the total number, the effective frequency and the effective linear and quadratic coupling constants of the low frequency modes coupled to the transition; σ_{in} reflects an eventual temperature independent inhomogeneous broadening.

We have recently obtained information on heme pocket dynamics of various heme proteins through the analysis of the Soret band in the temperature interval 20–300 K (Cordone et al. 1986, 1988; Leone et al. 1987; Cupane et al. 1988, 1993 a, b; Di Iorio et al. 1991; Di Pace et al. 1992). Interestingly, the thermal line broadening does not obey Eq. (1) in the whole temperature range investigated but only at low temperature, while at high temperature a σ^2 increase well above the prediction of Eq. (1) is observed (Di Pace et al. 1992; Cupane et al. 1993 a, b). This effect was attributed to the presence of non harmonic terms in soft nuclear motions coupled to the Soret transition. Moreover, for carbonmonoxy myoglobin, the σ^2 behavior was found to be in qualitative agreement with that for mean square displacement ($\langle x^2 \rangle$) of both the iron atom and non exchangeable hydrogens atoms, measured through Mössbauer (Parak et al. 1982) and neutron scattering spectroscopy (Doster et al. 1989) respectively. This agreement is both interesting and puzzling, since the different techniques employed probe different parts of the protein and motions occurring over different time scales. In order to show which motions are probed by our approach and to assess the role played by motions of the iron, we compare here the thermal line broadening of the Soret band of protoporphyrin IX, heme-CO and heme-OH with the ones for heme proteins. Results indicate that a central role in the thermal broadening of the Soret band is played by iron motions with respect to the porphyrin plane, thus suggesting that large scale protein soft motions, stemming from substate interconversions (Frauenfelder et al. 1988), are reflected in the iron dynamics, and that in turn brings about the observed broadening of the Soret band.

Materials and methods

Hemin (bovine, Lot n° 57F-0416) and protoporphyrin IX (Lot n° 293137-1189) were purchased from Sigma Chem-

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ical Co. (St. Louis, MO, USA) and Fluka Biochemika (Buchs, Switzerland) respectively, and were used without further purification. To prepare heme-CO, ferric hemin was dissolved in a suitable water + cosolvent + buffer mixture; this solution after equilibration with 1 atm. CO was treated with dithionite under strictly anaerobic conditions. Final samples for optical spectroscopy measurements contained $\approx 1.5\times10^{-6}$ M heme-CO; 2×10^{-2} M borate buffer pH 9 (in water at room temperature) and 3×10^{-4} M dithionite; solvent conditions were 98% v/v glycerol/water. The same procedure was followed for preparing heme-OH but skipping, of course, CO equilibration and addition of dithionite. Protoporphyrin IX was prepared by dissolving the powder in a solution containing 49% v/v methanol, 49% glycerol and 10^{-1} M NaOH. These solvent conditions were chosen for avoiding sample aggregation and having transparent samples down to low temperature; however, sample cracking occurred at a temperature of 60 K. For this reason measurements on protoporphyrin IX were performed only in the temperature range 70–300 K.

Details of the methods for optical measurements in the temperature range 10–300 K have been given in previous publications (Cordone et al. 1986; Di Pace et al. 1992; Cupane et al. 1993 a) and therefore are not reported here. The analysis of the Soret band profile as a function of temperature has been carried out according to Di Pace et al. (1992) and to Cupane et al. (1993 b); only the basic principles of the spectral deconvolution will be given here.

The Soret band profiles are described as the convolution of three terms:

$$A(\nu)=L(\nu)\otimes G(\nu)\otimes P(\nu)\,. \qquad (2)$$

The first term, $L(\nu)$, contains the usual Lorentzian lineshape for an absorption band; it takes into account the natural (homogeneous) width of the electronic transition together with its coupling with high frequency modes; according to this term the spectrum results from the superposition of a series of Lorentzians (one series for each high frequency mode): within each series the Lorentzians are spaced by multiples of the frequency of the vibrational mode (ν_j). We recall that we consider "high frequency modes" those for which $h\nu_j \gg K_B T$, i.e. those for which only transitions from the ground vibrational state can occur.

The coupling of the electronic transition with a 'bath' of low frequency modes introduces the second term, $G(\nu)$, in Eq. (2) and gives rise to a Gaussian line broadening. "Low frequency modes" are those for which $h\nu_j \leq K_B T$, i.e. those for which transitions can also start from excited vibrational levels. When the effects of coupling with both high and low frequency modes are taken into account, the spectral line shape is therefore given by a superposition of Voigtians (convolution of a Lorentzian with a Gaussian).

Further contributions to the spectral line width, originating from conformational heterogeneity (inhomogeneous broadening) due to the presence of conformational substates (Frauenfelder et al. 1988; Ormos et al. 1990) and different heme group environments, give rise to the third term, $P(\nu)$, in (2). Inhomogeneous broadening can be taken into account by assuming a distribution of the purely electronic transition frequency that is independent of temperature. In liganded derivatives the metal is locked in the heme plane by the ligand molecule and therefore the conformational distribution of heme group environments (assumed to be Gaussian) maps into a Gaussian distribution of spectral transition energies. The convolution with a further Gaussian term does not alter the overall shape or symmetry of the band but simply adds a constant term (σ^2_{in}) to the Gaussian line broadening.

Results and discussion

In Fig. 1 we show the 10 K Soret band profile of heme-OH, with the relative fitting performed according to the analysis proposed by Di Pace et al. (1992). The quality of the fitting is excellent, and it improved with increasing temperature. In Fig. 2 we report the temperature dependence of the Gaussian width (σ^2 values); the continuous lines represent fitting of σ^2 values to (1); fitting parameters are reported in Table 1. Figure 2 shows that only σ^2 values for protoporphyrin IX (i.e. in the absence of iron atom) can be fit in terms of (1) up to room temperature, while for heme-CO and heme-OH a suitable fitting in terms of (1) can only be performed up to $\approx$180 K, which can be safely assumed to be the solvent glass transition temperature (see e.g. Cordone et al. 1988)[1]. This behavior, al-

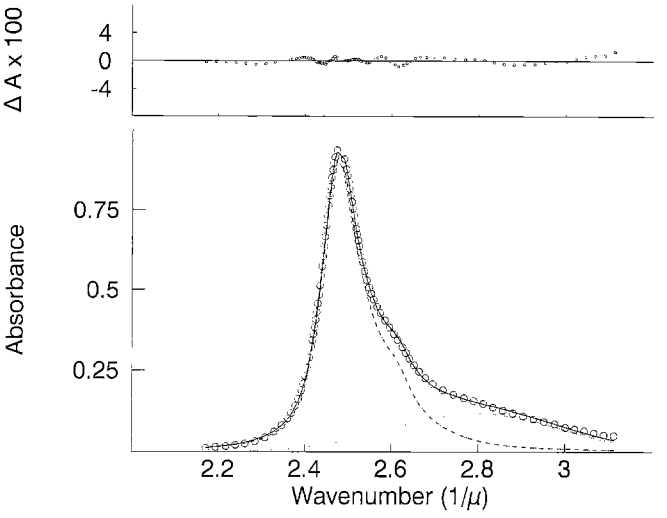

Fig. 1. Soret band of heme-OH at 10 K. *Circles:* experimental points; *broken lines:* fitting performed according to Eq. (3) of Di Pace et al. (1992) and Gaussian extrapolation that takes into account contributions from the nearby N band; continuous line: overall synthesized band profile. For the sake of clarity not all the experimental points have been reported. The residuals between the experimental and the reconstructed spectrum are reported in the upper panel

[1] Besides the fact that fitting in terms of (1) in the whole temperature range gives a sizeable systematic misfit, an unambiguous indication that (1) can fit data for heme-CO and heme-OH only to $\approx$180 K, arises from the fact that for $T\rightarrow\infty$ Eq. (1) results in an expression, linear in T, whose extrapolation to 0 K gives σ^2_{in} (i.e. either positive or zero values). On the contrary, the linear extrapolation to 0 K of the high temperature σ^2 values, for heme-CO and heme-OH gives negative, non physical, inhomogeneous broadening

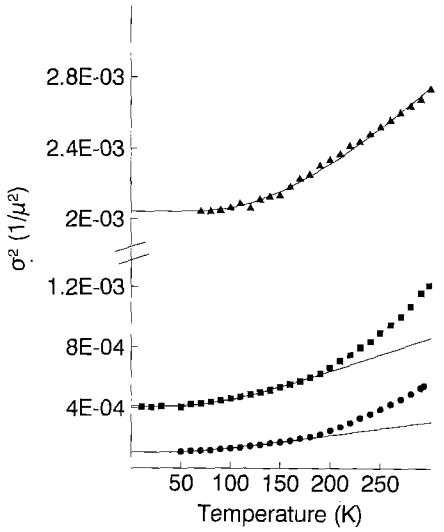

Fig. 2. σ^2 vs. temperature for protoporphyrin IX (*triangles*), heme-OH (*squares*) and heme-CO (*circles*). Continuous lines represent fittings in terms of Eq. (1) in the text. Note that for heme-CO and heme-OH fittings are performed only up to 180 K

Table 1. Values of parameters that characterize the coupling of the Soret band with low frequency motions

	NS_I [a]	$\langle\nu\rangle$/cm^{-1}	σ_{in}/cm^{-1}
Heme-CO	0.5±0.1	146±10	–
Heme-OH	0.8±0.2	194±10	97±8
Protoporphyrin IX	1.2±0.3	347±15	234±10
Hb[b]	0.6±0.1	140±10	–
SWM[b]	0.7±0.1	110±10	–
HbCO[c]	0.5±0.2	170±20	70±23
SWMbCO[c]	0.3±0.2	180±30	53±38

[a] NS_I values were obtained under the assumption that $R_I^2 \approx 1$
[b] Data taken from Cupane et al. 1993a
[c] Data taken from Di Pace et al. 1992

ready reported for different heme proteins (Di Pace et al. 1992; Cupane et al. 1993a, b), led us to suggest that Eq. (1) is not obeyed in the whole temperature range investigated since non harmonic contributions to nuclear motions become evident on increasing the temperature; such contributions ($\Delta\sigma^2$ values, i.e. the differences between the experimental σ^2 values and the continuous lines in Fig. 2) are reported in Fig. 3. We stress that the different behavior of protoporphyrin IX with respect to heme-CO and heme-OH cannot be attributed to the different solvent conditions (see "Materials and methods"): indeed, a control experiment performed with heme-OH in 49% methanol + 49% glycerol gave results essentially identical to those reported in Fig. 2.

From the data shown in Fig. 2 we infer that the anharmonic contributions we observe must be ascribed to motions of the iron with respect to the porphyrin plane. Plausibly, after melting of the solvent medium, the release of constraints imposed by the external matrix enables iron-ligand motions of amplitude larger than that expected from the low temperature behavior. We think it interesting to note that the average frequencies of vibrational modes (in

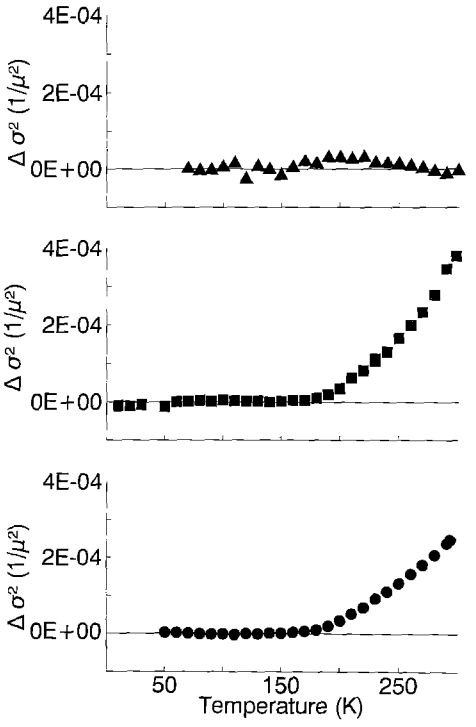

Fig. 3. $\Delta\sigma^2$ values as a function of temperature; symbols as in Fig. 2. $\Delta\sigma^2$ is defined as $\Delta\sigma^2 = \sigma^2_{exp} - \sigma^2_{theor}$, where σ^2_{exp} is given by the data points in Fig. 2 and σ^2_{theor} is given by Eq. (1); in other words, $\Delta\sigma^2$ is the difference between data points and continuous lines in Fig. 2

the harmonic region) are 146 cm^{-1} and 194 cm^{-1} for heme-CO and heme-OH respectively. This difference agrees with the different ligand masses; accordingly, high temperature deviations from harmonic behavior are larger for the OH derivative.

For protoporphyrin IX, the Gaussian line broadening is almost an order of magnitude larger than that for the hemes; moreover, harmonic behavior, characterized by larger average frequency and linear coupling constant with respect to heme-CO and heme-OH, is observed in the whole temperature range. We attribute these harmonic motions to "out-of-plane" porphyrin deformation modes (Spiro 1983); in heme complexes the presence of the metal probably hinders such motions and therefore their role in the Soret band thermal behavior. It should be mentioned that the harmonic behavior of the thermal line broadening observed for protoporphyrin IX does not necessarily imply that motions that bring about anharmonic contributions are absent in this system, but rather that, owing to the absence of the iron atom, their coupling with the Soret band is so low as to make them no longer detectable.

On the basis of the above conclusions we infer that, also in heme proteins, motions of the iron with respect to the porphyrin plane must play an important role in determining the observed thermal broadening of the Soret band (Di Pace et al. 1992; Cupane et al. 1993a, b); $\Delta\sigma^2$ values for deoxy and CO-bound spermwhale myoglobin are reported in Fig. 4. In this case the anharmonic iron motions, being driven by the protein matrix via the iron-proximal histidine bond, plausibly reflect interconversion among sub-

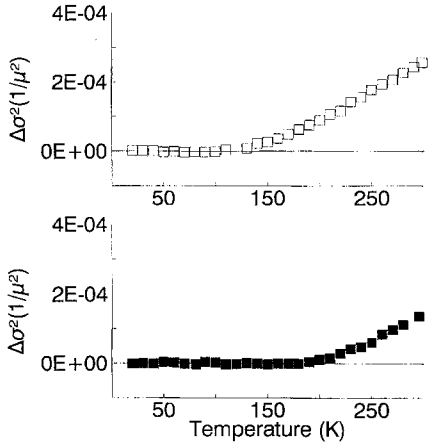

Fig. 4. $\Delta\sigma^2$ values relative to carbonmonoxy (*lower panel, full symbols*) and deoxy (*upper panel, open symbols*) derivatives of sperm-whale myoglobin (data taken from Cupane et al. (1993 b)

states of suitable tiers (Frauenfelder et al. 1988). In this respect it is worth noting that $\Delta\sigma^2$ values are smaller when the heme iron is embedded in the protein (compare Figs. 3 and 4, lower panel), in agreement with the expectation that the protein matrix introduces limitations to the iron "freedom".

Alternative models could be put forward to explain the anharmonic σ^2 behavior observed in heme complexes and in heme proteins. These could consider that a central role is to be ascribed to motions of the bound ligand and/or of residues in the distal side of the heme pocket (like, for example, the distal histidine). Although such contributions may be present and relevant, as demonstrated by the modulation of the σ^2 thermal behavior brought about by different bound ligands (see Fig. 2) or by the conformation of the distal heme pocket (Cupane et al. 1993 b), the centrality of iron motions is borne out a) by the presence of anharmonic contributions also in deoxy derivatives of heme proteins (Cupane et al. 1993 a) and b) by the fact that in proto myoglobin (i.e. in myoglobin reconstituted with protoporphyrin IX but lacking the iron atom) the σ^2 temperature dependence is harmonic up to 270 K (unpublished results).

A further interesting point concerns the comparison between deoxy and CO hemeprotein derivatives. It has been shown (Cupane et al. 1993 a, b) that in deoxy derivatives $\Delta\sigma^2$ values start to be sizeable at about 110 K, while in CO derivatives this happens at about 180 K (Di Pace et al. 1992; see also Fig. 4): on the basis of the present results one can conclude that, in the absence of ligand, anharmonicities in iron motions modulated by the protein matrix, are already relevant at 110 K. In ligated proteins the presence of the CO molecule hinders this effect by "bridging" the proximal to the distal side of the protein, which therefore has to "wait for" the softening of the external matrix (glass transition), in order to undergo interconversion among the substates relevant to the observed effect.

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