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Influence of Static and Dynamic Disorder on the Visible and Infrared Absorption Spectra of Carbonmonoxy Horseradish Peroxidase

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ABSTRACT Spectroscopy of horseradish peroxidase with and without the substrate analog, benzohydroxamic acid, was monitored in a glycerol/water solvent as a function of temperature. It was determined from the water infrared (IR) absorption that the solvent has a glass transition at 170–180 K. In the absence of substrate, both the heme optical *Q*(0,0) absorption band and the IR absorption band of CO bound to heme broaden markedly upon heating from 10–300 K. The *Q*(0,0) band broadens smoothly in the whole temperature interval, whereas the IR bandwidth is constant in the glassy matrix and increases from 7 to 16 cm^{-1} upon heating above the glass transition. Binding of substrate strongly diminishes temperature broadening of both the bands. The results are consistent with the view that the substrate strongly reduces the amplitude of motions of amino acids forming the heme pocket. The main contribution to the *Q*(0,0) bandwidth arises from the heme vibrations that are not affected by the phase transition. The CO band thermal broadening stems from the anharmonic coupling with motions of the heme environment, which, in the glassy state, are frozen in. Unusually strong temperature broadening of the CO band is interpreted to be caused by thermal population of a very flexible excited conformational substrate. Analysis of literature data on the thermal broadening of the A_0 band of Mb(CO) (Ansari et al., 1987. Biophys. Chem. 26:337–355) shows that such a state presents itself also in myoglobin.

INTRODUCTION

Globular proteins must maintain a three-dimensional structure and simultaneously exhibit conformational flexibility. This is true for all proteins, but the relationship between dynamics and protein function has received much attention in heme proteins. Kinetic measurements of CO recombination in myoglobin after photolysis has lead to the view of conformational substrates and energy barriers (Frauenfelder et al., 1994). Whereas the properties of hemoglobin and myoglobin have been extensively studied, a comparison with other heme proteins is one way to examine how protein dynamics vary, how physical parameters of the heme are influenced by these fluctuations, and how important for the protein functioning their dynamics are.

From this point of view, horseradish peroxidase (HRP) can give an interesting perspective. One major difference from hemoglobin and myoglobin is that a variety of different bulky substrates can bind to HRP. On the basis of x-ray analysis it was concluded that the aromatic substrate analog, benzohydroxamic acid (BHA), binds in a hydrophobic pocket near the heme crevice, but that little structural rearrangement is seen in the amino acids directly involved in binding to the heme (Henriksen et al., 1998). However, BHA can affect the heme in several ways other than direct contact. In the substrate-free enzyme there is a channel from the aqueous solution to the heme group (Gajhede et al., 1997). BHA seems to extend into the channel, and the location and coordination of waters are also influenced upon substrate binding around the heme (Henriksen et al., 1998). Therefore, it follows that it may influence the heme by changing the amplitudes of various distortional modes of the heme periphery, and also the positional fluctuations of the surrounding amino acids and water molecules. Because the fluctuating amino acids and water molecules produce a varying internal electric field in the heme crevice (Kushkuley and Stavrov, 1997; Laberge et al., 1999), a change in these fluctuations can be expected to affect the observed spectral line widths. BHA, with its own charge distribution, may also directly affect the spectral properties of the heme by coulombic effects (Kaposi et al., 2001).

In this study we are discussing the use of the spectral features of the CO-heme complex to study the dynamics of HRP. Particular attention is given to the effect of substrate and the interactions of the protein with the glycerol/water solvent. The CO complex of ferrous HRP is low spin and has two very well known spectroscopic markers: the optical *Q*(0, 0) band and the infrared (IR) stretch of CO. The *Q*(0, 0) optical band is formed by the $S_0 \rightarrow S_1$ electronic $\pi \rightarrow \pi^*$ transition of the heme (Gouterman, 1978). The CO IR absorption band corresponds mainly to the stretching C-O vibration. Their positions and widths are controlled by the protein environment of the heme (Gouterman, 1978; Park et al., 1991; Ray et al., 1994; Kushkuley and Stavrov, 1996, 1997; Phillips et al., 1999; Manas et al., 1999). Therefore,

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they can be used as probes of both the structure and dynamics of the heme pocket.

The hypothesis is that at high temperature there is a wider distribution of conformations that will produce a broadening of both these spectral marker bands. As temperature decreases, the protein adiabatically relaxes to lower energy states, and the band should narrow. At lower temperatures (170–180 K for glycerol/water) the solvent forms a glass, protein large-scale motions are damped, and the protein retains the conformations that it exhibits at the temperature of the glass formation. We are asking whether the two spectral markers are affected by the protein and the solvent matrix in the same way. The experimental data are interpreted using quantum chemical calculations (QCC), vibronic theory of activation (VTA), method of moments, and general theory of optical absorption band shape.

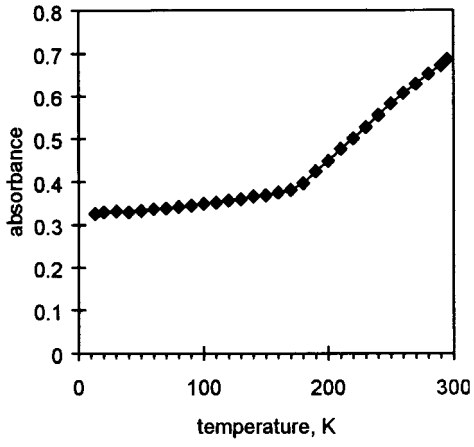

FIGURE 1 Temperature dependence of OH stretch. HRP concentration: 2.4 mM in 40% D_2O and 60% deuterated glycerol. Absorbance, arbitrary units, at 3400 cm^{-1}.

MATERIALS AND METHODS

Materials

Water was deionized and then glass-distilled. Sodium dithionite and HRP type C was obtained from Sigma Chemical Co. (St. Louis, MO). HRP was purified as described by Kaposi et al. (1999) based upon the procedure of Paul (1958). The CO derivative of HRP was prepared as follows. The solvent (60% glycerol/water v/v) was aspirated to remove dissolved gases and 300 ml was placed in a 25-ml round bottom boiling vessel fitted with a vertical extension with an offset loop to contain dry components and a valve to close the vessel from the atmosphere. Lyophilized HRP (5 mg) and 5 mg of solid sodium dithionite were placed in the loop. CO was introduced to the vessel for ~15 min at 8–10 psi. After 15 min, the dry components were introduced to the solution and mixed by swirling. The solution became bright orange/red and this color deepened in intensity over time. The solution was allowed to remain in the CO atmosphere for 30 min, after which the vessel was opened and the sample was immediately placed into the cell holder. The final pH was adjusted to be 6.0.

Spectroscopy

IR spectra were obtained with a Bruker IFS 66 Fourier transform IR instrument (Bruker, Brookline, MA) as described by Kaposi et al. (1999). A Hitachi PerkinElmer (PerkinElmer Instruments, Shelton, CT) absorption instrument was used to take the visible absorption spectra. For both instruments, a transmission cell holder with CaF_2 windows was used to hold the sample. The path length was 100 μm for the optical experiments and 200 μm for the IR measurements. The temperature of the sample was regulated by a top-loading OmniPlex cryostat (APD Cryogenics, Allentown, PA). The spectra were obtained in the sequence of high temperature to low temperature at 10° increments starting at 290 K. The rate of cooling was ~1°/min. The lowest temperature obtained was 11–13 K. In some experiments, absorption spectra were again taken as the temperature warmed up. No irreversible effects were seen.

Glass-liquid phase transition

To determine the solvent glass-liquid phase transition, the OH stretching frequency of the solvent water was measured. OH is hydrogen-bonded to other groups, and the strength of the hydrogen bonds in general increases as temperature decreases. As the strength increases, the band shifts to lower frequency (Jeffrey, 1997). The OH stretching band of HOH, centered at ~3400 cm^{-1}, is a composite band which consists of antisymmetric and symmetric stretching modes. However, for HOD in a predominantly DOD solution, the composite OH stretches are decoupled and the resulting band from DOH arises from a simple OH stretch. Therefore, to determine the glass-liquid transition, the sample was suspended in D_2O and deuterated glycerol. The residual water in the protein and the solvent gave rise to the OH stretching band. There was a shift to lower frequency in this band as the temperature decreases, consistent with increased hydrogen bonding with lowering temperature. Plotting the peak intensity of the band allows us to identify the glass transition at $T_c = 170–180$ K (Fig. 1).

QCC

QCC of the Fe(II)(P)(Im)(CO) electronic structure, population of *i*-th orbital of CO (q_i), and their dependence on the electrostatic interactions are performed using the intermediate neglect of differential overlap (INDO) version of molecular orbital (MO)-linear combination of atomic orbitals approach (Bacon and Zerner, 1979; Anderson et al., 1986; Edwards et al., 1986; Du et al., 1991). This type of approach is based on the self-consistent solution of the Hartree-Fock equation with the inclusion of all the one-center exchange terms necessary for rotational invariance and accurate spectroscopic predictions, as well as an accurate description of integrals involving three-dimensional atomic orbitals. In the INDO technique the one-center core integrals are obtained only from ionization potentials. In this work the spin-restricted Hartree-Fock method is used and the calculations were performed using Mataga-Nishimoto parameterization. For the INDO QCCs, the ZINDO program, kindly supplied by Dr. M. Zerner, Department of Chemistry, University of Florida, is used.

To calculate orbital electron density transfers to and from CO the MOs obtained by the INDO calculations are rewritten in the basis of the eigenfunctions of the free CO and atomic functions of other atoms. Then the occupations of the CO eigenfunctions in the complex are calculated as the Mulliken population of the corresponding orbitals.

Chemical VTA by coordination

Vibronic theory of chemical activation by coordination is described in detail elsewhere (Stavrov et al., 1993; Kushkuley and Stavrov, 1996, 1997). Its main idea is to make a bridge between the widespread MO description of the electronic structure, as well as the coupling of electronic states with the nuclear configuration described by the vibronic coupling theory.

In particular, it was shown using VTA that the following expression for the force field constant of the normal vibration Q in the γ-th electronic state can be written:

$$K^{\gamma} = \sum_{i} q_i^{\gamma} k_i, \tag{1}$$

in which q_i^{γ} is the population of i-th orbital in the γ-th electronic state and k_i is the second-order orbital vibronic constants. It follows from this expression that the dependence of the force field constant K^{γ} on the structure of any compound in any γ electronic state is mainly controlled by the MO populations, q_i^{γ}.

Spectral analysis

Spectral analysis is performed using method of moments. Zero, first, and second moments of a band are (Lax, 1952):

$$M_o = \int F(\omega) d\omega$$

$$M_1 = \frac{1}{M_0} \int \omega F(\omega) d\omega \tag{2}$$

$$M_2 = \frac{1}{M_0} \int \omega^2 F(\omega) d\omega - {M_1}^2.$$

Zero moment equals the intensity of the band, the first moment is a center of gravity of the band, and second moment describes its width. In the case of a Gaussian band, the first moment coincides with the band maximum position, whereas

$$M_2^G = \frac{\Gamma^2}{8 \ln 2}, \tag{3}$$

where Γ is the full width at half maximum of the band.

Temperature dependence

Temperature dependence of the first three moments of the absorption bands is interpreted using of theory of vibrational structure of optical absorption band (Lax, 1952; Markham, 1959; Perlin, 1964; Rebane, 1970). The simplest results were obtained in the Condon approximation (the dipole transition moment does not depend on the nuclear coordinates) for a transition between the ground and excited electronic states, which are linearly shifted one with respect to another along a number of normal coordinates. It was shown that in this case the zero and first moments of the band (or number of bands, corresponding to the same electronic transition) do not depend on temperature, whereas the second moment has a very specific temperature dependence:

$$M_2 = A_1 + \sum_{i} B_i^2 \frac{\hbar}{2 m_i \omega_i} \coth\left(\frac{\hbar \omega_i}{2kT}\right). \tag{4}$$

In Eq. (4) A_1 is the temperature independent contribution, which stems from an inhomogeneous distribution or some other mechanisms and is always non-negative; B_i is a linear constant of electron-vibrational interaction with i-th normal coordinate, which causes shift of the excited state in respect to the ground one in the i-th direction; and ω_i and m_i are frequency and reduced mass of a vibration along this normal coordinate.

The weak or moderate dependence of dipole transition moment on nuclear coordinates causes a temperature dependence of M_0 and M_1, whereas the temperature dependence of M_2 has the same form as in Eq. (4) (Markham, 1959).

In the case of electron-vibrational coupling with high-frequency vibration ($h\nu \gg kT$) the light absorption corresponding to one electronic transition usually leads to a number of bands (Rebane, 1970). These bands are the zero-(high-frequency) vibration band and a number of satellites; each satellite corresponds to excitation of the respective number of high-frequency vibrations. Eq. (4) describes temperature dependence of the second moment of all of these bands together and not of each of them. However, each such band is formed by electron-vibrational interaction with the same lower-frequency vibrations. Therefore, they have close shapes, this phenomenon being called a similarity law (Rebane, 1970). Hence, M_2 of each band can be described by the same Eq. (4), where contributions of only lower-frequency vibrations are taken into account. The visible spectra of the proteins under consideration represent a nice example of the spectrum, described above, with the $Q(0, 0)$ band being the zero-(high-frequency) vibration band. Thus, M_2 of this band can be described by Eq. (4) (Sanfratello et al., 2000), in which summation is over lower-frequency vibrations.

To fit the M_2 temperature dependence, the sum over contributions from different vibrations is substituted for interaction with one vibration with an effective frequency of ω and reduced mass of m (Cupane et al., 1988)

$$M_2 = A_1 + \frac{B_1}{\hbar \omega} \coth\left(\frac{\hbar \omega}{kT}\right), \tag{5}$$

where

$$B_1 = \frac{\hbar^2}{2m} \sum_{i} B_i^2. \tag{6}$$

It was shown, using double adiabatic approximation (Rebane, 1970), that temperature dependence of an IR band can be interpreted using the theory developed for the optical absorption spectra. Consequently, Eqs. (4) and (5) are valid for describing the temperature dependence of an IR absorption band if two criteria are satisfied: 1) the excited state of the active vibration is weakly populated; and 2) there is linear anharmonic coupling of the active vibration to lower frequency vibration(s).

RESULTS

HRP(CO) visible absorption spectra

Visible absorption spectra of the HRP(CO) complex in glycerol/water are shown in Fig. 2 for the temperature range of ~12–290 K. The spectra show two bands in visible region, typical of low-spin hypsoporphyrins (Gouterman, 1978). The peak maximum of the Soret (not shown) is at 423 nm and the two visible absorption bands are at 541 nm (18,484 cm^{-1}) and 572 nm (17,470 cm^{-1}), respectively, are the same as reported previously at room temperature for this compound (Tamura et al., 1972). As temperature is lowered, the $Q(0,0)$ band becomes narrower, with a small shift in absorption maximum to lower frequency.

Absorption spectra of the HRP(CO)-BHA complex in glycerol/water are shown in Fig. 3 for the temperature range of ~12–280 K. The presence of BHA causes a split in the band spectra as was shown in a previous paper (Kaposi et al., 2001). This work also showed that a split in the band occurred upon binding naphthohydroxamic acid. A pronounced effect of binding this same substrate on the Q band splitting of Mg-mesoporphyrin-HRP was studied and discussed earlier by Balog et al. (1997, 2000).

It is well known (Gouterman, 1978) that the Q band borrows intensity from the B band because of the electron-

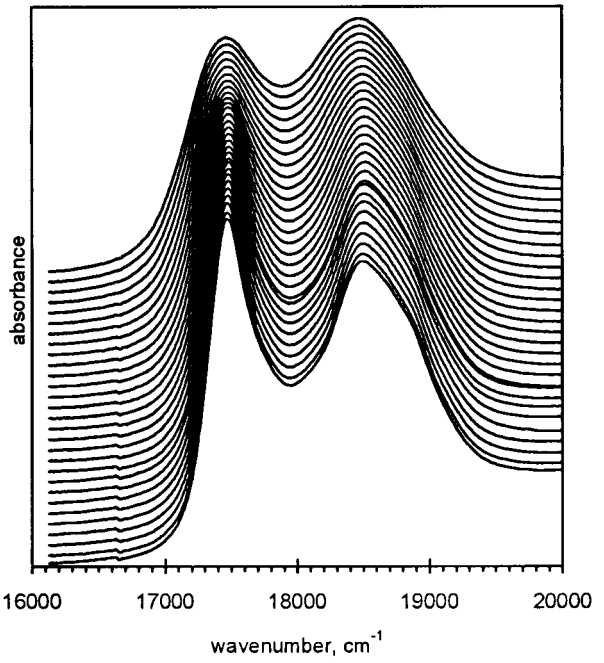

FIGURE 2 Optical absorption of CO-HRP at pH 6.0 in glycerol/H_2O. Temperature from top to bottom: 290, 280, . . . , 30, 20, and 12 K.

vibrational interaction with high-frequency vibrations. This interaction, for example, causes appearance of the $Q(0-1)$ satellite. It hardly contributes to the Q band shape temperature dependence, because vibrational quanta of these high-frequency vibrations are >1000 cm^{-1}. Therefore, band

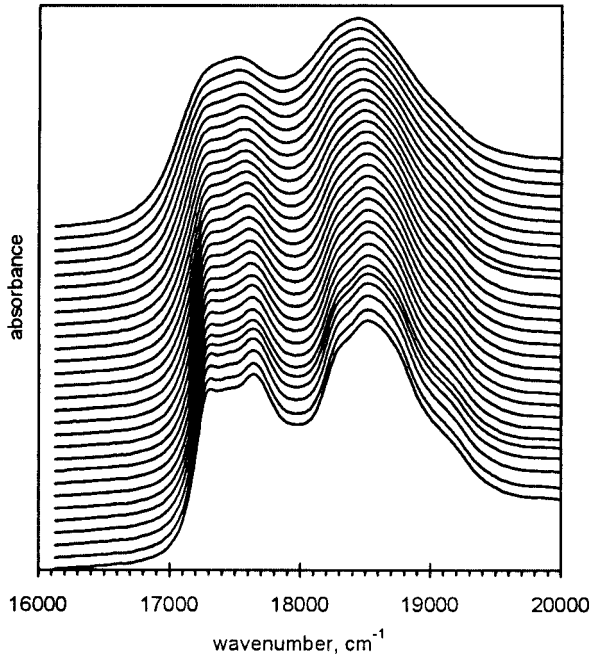

FIGURE 3 Optical absorption of CO-HRP-BHA at pH 6.0 in glycerol/H_2O. Temperature from top to bottom: 290, 280, . . . , 30, 20, and 12 K.

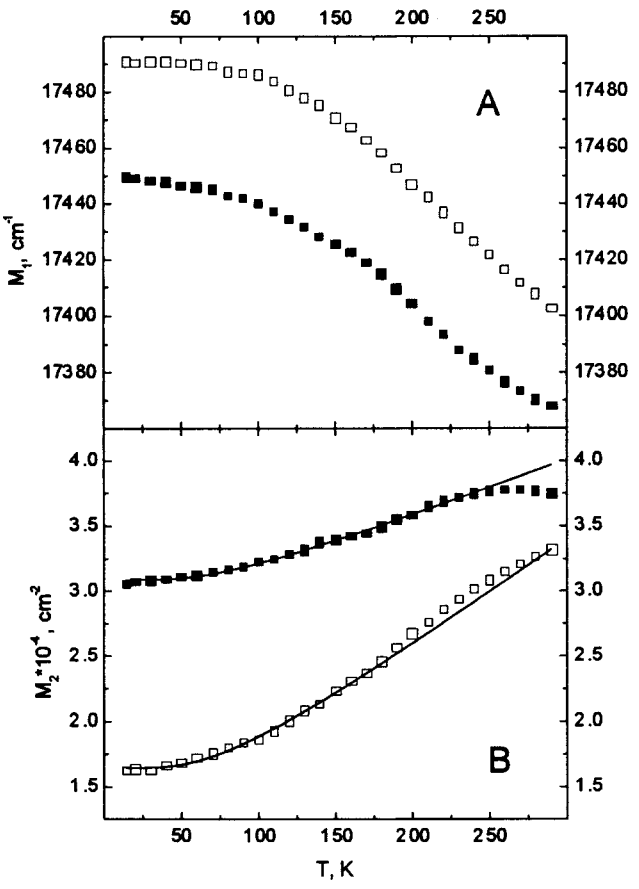

FIGURE 4 Temperature dependence of M_1 (*A*) and M_2 (*B*) of the optical absorption $Q(0, 0)$ band of HRP(CO) (□) and HRP(CO)-BHA (■): experimental data and fit of the experimental data at $T < T_c$ using Eq. (5) (*solid curve*). The data are determined from the spectra shown in Figs. 2 and 3. Parameters for HRP(CO) and HRP(CO)-BHA are respectively $A_1 = 0.7 \cdot 10^4$ and $2.6 \cdot 10^4$ cm^{-2}; $B_1 = 1.4 \cdot 10^6$ and $0.7 \cdot 10^6$ cm^{-3}; and $h\omega = 150$ cm^{-1}.

shape of each satellite is controlled by the low-frequency vibrations for which Condon approximation can be used. Consequently, the Eqs. (4)-(6) can be used to interpret the experimentally observed temperature dependence of M_2. This conclusion is supported by the experimental data on M_1 (Fig. 4 *A*) and M_0 (not presented), which show that their temperature dependencies are weak.

The experimental data obtained for both compounds in the glassy state ($T < T_c$) were successfully fitted using Eq. (5), the results of the fitting procedure being presented on Fig. 4 *B*, and the parameters are presented in the Fig. 4 figure legend.

Temperature dependence of the CO IR absorption band of HRP(CO)

The IR absorption band of CO bound to ferrous HRP is located at $\nu(CO) = 1903$ cm^{-1} (Fig. 5 *A*). This value

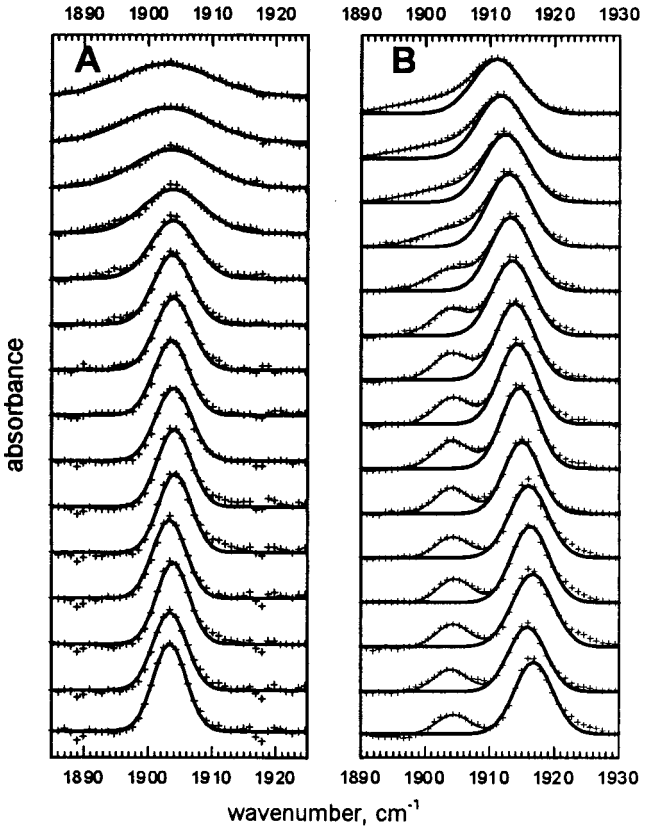

FIGURE 5 Temperature dependence of the IR absorption of CO bound to Fe(II)HRP. Protein concentration: 2.4 mM. Solvent was glycerol/H_2O. Temperature from top to bottom: 290, 270, . . . , 50, 30, 12 K (*A*) Substrate-free protein. Solid line is the Gaussian fit to the datapoints. (*B*) Protein with substrate. BHA was at ~3.0 mM. Thick line shows the Gaussian component of the major peak, and thin line is the fit to two Gaussian components.

reproduces the value within 1 cm^{-1} observed by previous workers for the enzyme in aqueous solution (Barlow et al., 1976; Smulevich et al., 1986; Uno et al., 1987). The protein, purified as described in Methods, shows a single IR absorption band within the resolution of the spectrum. This peak could be fit to a single Gaussian function, as indicated by the good agreement between the datapoints and the solid line, which represents the fitted curve. As temperature is reduced from 290 K to 12 K, full width at half maximum of the CO absorption band decreases from 16 to 7 cm^{-1}, but the position of the peak remains at 1903 cm^{-1}.

The CO band for the HRP(CO)-BHA tertiary complex is given in Fig. 5 *B*. The absorption maximum at 290 K is at 1911 cm^{-1}, which is in agreement with a previous study (Uno et al., 1987). The peak narrows from 9 to 6 cm^{-1}, full width at half maximum, in going from 290 to 20 K. A peak seen at 1903 cm^{-1} has the same position and temperature dependence as the substrate-free protein (Fig. 4 *A*). Therefore, this peak likely arises from some protein molecules that are not substrate bound. Consequently, the spectra were

TABLE 1 Spectral Features of CO-Fe(II)HRP

Conditions	T K	ν_{CO} cm^{-1}	FWHM cm^{-1}	$Q(0,0)$ cm^{-1}	FWHM cm^{-1}
Glycerol/H_2O	290	1903	16	17375	505
	12	1903	7	17450	280
glycerol/H_2O BHA	290	1911	9	N.D.	N.D.
	12	1916	6	N.D.	N.D.

N.D.: Not determined because of a split origin.

fitted to two Gaussians, the low energy one had position and width equal to those of the free BHA protein. The spectral features are summarized in Table 1.

HRP(CO) manifests a low frequency compared with other heme proteins, ν(CO) = 1903 cm^{-1}. Evangelista-Kirkup et al. (1986) and Smulevich et al. (1986) suggested that this frequency reduction is caused by the hydrogen bond between the proximal histidine and aspartate (Asp235). To check this assumption we used INDO QCC (see Materials and Methods) to determine the effect of the formation of this bond on the electron density transfer to and from coordinated CO. The presence of a carboxyl oxygen atom participating in the hydrogen bond was simulated by location of a unit negative charge at 2.9 Å from the distal nitrogen of the proximal histidine (N_δ). It follows from the calculations, that the hydrogen bond increases the population of the antibonding 2π CO orbital by 0.06 e^-, population of the 5σ CO orbital being hardly affected. Using VTA theory we ascertained that this increase in the π backbonding is expected to decrease ν(CO) by 29 cm^{-1}. This result shows that formation of the hydrogen bond mainly contributes to the ν(CO) reduction.

ν(CO) is much higher than the energy of thermal motion at room temperature. Thus, its excited vibrational states are hardly populated even at room temperature and barely contribute to the formation of the corresponding IR absorption band. We assume that the anharmonic coupling is weak enough to be reliably described in the linear approximation (Rosenfeld and Stavrov, 1994; Bitler and Stavrov, 1999). This assumption is strongly supported by the experimentally observed independence of M_1 of the CO band of HRP(CO) of temperature (see Fig. 6 *A*). Independence of temperature of M_0 (not shown) indicates also that the Condon approximation must reliably describe the temperature dependence of the IR CO band of CO complexes of HRP. Consequently, both criteria mentioned in Methods are fulfilled. Hence, the results of theory of vibrational structure of optical absorption band and, in particular, Eqs. (4)–(6) can be used to interpret temperature dependence of the first three moments of the IR CO band. In these equations, B_i is a constant of the linear anharmonic coupling of the CO vibration to some other *i*-th vibration. It can be a vibration of the heme or its environment (protein amino acids or solvent molecules).

However, both the experimental temperature dependencies of M_2 of HRP(CO) and HRP(CO)-BHA (Fig. 6) cannot

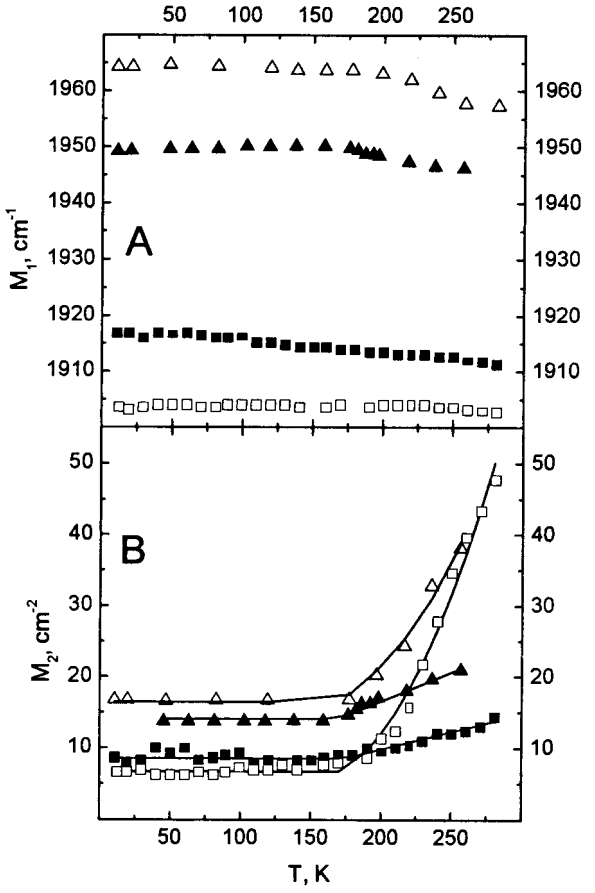

FIGURE 6 Temperature dependence of the first (*A*) and second (*B*) moments of the IR CO absorption band: experimental data on HRP(CO) (□), HRP(CO)-BHA (■), A_0 band of Mb(CO) (△), and A_1 band of Mb(CO) (▲), and fit, obtained using Eq. (9) for A_1 band of Mb(CO) and HRP(CO)-BHA data and Eq. (10) for A_0 band of Mb(CO) and HRP(CO) (*solid curves*). The fitting parameters are presented in Table 2.

be fitted in the frameworks with Eq. (5) without invoking some additional mechanism, contributing to the CO band broadening.

Therefore, we assumed that the properties and, in particular, CO band shape of HRP are affected by the glass-liquid phase transition. This assumption is strongly supported by the fact, that M_2 is constant at $T < T_c$ and sharply increases upon heating at $T > T_c$ (Fig. 6 *B*). Therefore, M_2 temperature dependence must be assigned to the interaction with large amplitude (low-frequency) motions of the heme environment, which are controlled by the glass-liquid phase transition. Upon cooling the sample below T_c, these vibrations are frozen in into the glassy matrix and M_2 remains constant, being equal to its magnitude at T_c. In the liquid state at $T > T_c$ amplitudes of these vibrations increase upon heating, increasing M_2. To express mathematically this effect of the phase transition, one can

TABLE 2 Magnitudes of Parameters Describing Temperature Dependence

	T_c K	B_{gr} cm^{-2}	B_{ex} cm^{-2}	ΔE cm^{-1}
HRP(CO), pH 6.0	175	0.014	4.2	638
HRP(CO)-BHA, pH 6.0	175	0.049	N.D.	∞
Mb(CO), A_0, pH 5.0	170	0.090	5.4	794
Mb(CO), A_1, pH 6.8	170	0.084	N.D.	∞

N.D.: Not determined because the excited conformational substate is hardly populated.

Magnitudes of the parameters describing temperature dependence of the IR CO absorption bands of carbonmonoxy complexes of horseradish peroxidase and myoglobin in different conditions.

replace real temperature by the effective one, T_{eff}, which equals T_c at $T < T_c$ and T at $T > T_c$:

$$T_{eff} = \frac{1}{2}\{[\mathrm{sign}(T_c - T) + 1]T_c + [\mathrm{sign}(T - T_c) + 1]T\}. \quad (7)$$

For the low-frequency vibrations the inequality $h\omega < kT_c$ is satisfied. Being interested in the M_2 behavior only at $T > T_c$, we use the equation

$$\coth\left(\frac{\hbar\omega_i}{2kT}\right) \approx \frac{2kT}{\hbar\omega_i}, \quad (8)$$

and obtain from Eq. (4) that in harmonic approximation

$$M_2 = A + kT\sum_i \frac{B_i^2}{m_i\omega_i^2} = A + BT_{eff}. \quad (9)$$

Temperature dependence of the HRP(CO)-BHA M_2 is successfully fitted using Eq. (9), parameters of the fit being presented in Table 2.

Taking into account that $A \geq 0$, one obtains from Eq. (9) that heating of HRP(CO) from T_c (170–180 K) to room temperature (300 K) can cause, at most, a 70% increase in M_2. The fact that this heating causes an increase of approximately seven times (see Fig. 6 *B*) unequivocally shows that the main contribution to the band broadening at $T > T_c$ stems from the coupling with strongly anharmonic motions of the heme environment.

The simplest way to understand the origin of this anharmonicity is to assume that the heme environment can exist in two energetically close states, the ground and excited (Doster et al., 1989). In each state low-frequency motions can be described as harmonic vibrations, but the *B* values are different in the ground and excited states, B_{gr} and B_{ex}, respectively. The independence of M_1 on temperature (Fig. 6 *A*) implies that the population of the excited substate does not change the position of the CO band, and, consequently, both substates are characterized by equal magnitudes of ν(CO).

Denoting the energy gap between these two states by ΔE and assuming for simplicity that $A_1 = 0$ (this assumption means, that the bandwidth in the glassy matrix is caused by a distribution of the heme environment along coordinates of the same large-amplitude motions), one obtains

$$M_2 = \frac{1}{1 + \exp\left(- \frac{\Delta E}{kT_{\text{eff}}}\right)}\left[B_{\text{gr}} + B_{\text{ex}}\exp\left(- \frac{\Delta E}{kT_{\text{eff}}}\right)\right]T_{\text{eff}}. \tag{10}$$

The experimental data were fitted using Eq. (10), the obtained magnitudes of parameters being presented in Table 2.

Ansari et al. (1987) studied the temperature dependence of M_2 of the A_0 (at pH 5.0) and A_1 (at pH 6.8) bands of complex sperm whale myoglobin with CO (MbCO). Their results also can be fitted using general theory of band shape and assuming that the glass-liquid phase transition freezes in large-amplitude motions of the protein. Temperature dependence of the A_1 band can be fitted using Eqs. (7)-(9), whereas interpreting the A_0 band temperature dependence we had to assume that an excited state is located close to the ground one. Weak temperature dependence of the A_0 band M_1 (Fig. 6 *A*) justifies usage of the Eq. (10) in this case also. The results of the fitting procedure and magnitudes of the fitting parameters are presented on Fig. 6 *B* and Table 2, respectively.

One must be very careful about the magnitudes of parameters obtained in the fitting procedure. For example, it was assumed in Eq. (10) that entropies of the ground and excited states are equal, the probability of their population being controlled only by the energy gap between them. However, the entropy of the excited state can be very different from the ground one. Unfortunately, the fitting procedure does not allow to determine separately magnitudes of ΔS, ΔE, B_{gr}, and B_{ex}. A change in the former leads to corresponding change in the latter: the larger the ΔS, the larger the ΔE, and smaller the B_{ex}. Therefore, the obtained ΔE magnitude by no means can be considered a reliable determination of the energy gap magnitude.

DISCUSSION

Q(0, 0) absorption band

It follows from the results of the fitting procedure that the temperature-independent part of M_2 (A_1) of HRP(CO)-BHA is ~3.5 times larger than that in HRP(CO). This difference is caused by splitting of the $Q(0, 0)$ band ($d = 320\ \text{cm}^{-1}$) in the protein with BHA (Kaposi et al., 2001). The components of the $Q(0, 0)$ band are Gaussians of approximately equal intensity (Fig. 3). It is simple to show that in this case the splitting increases M_2 by $(\delta/2)^2$. Subtraction of this contribution from the value, obtained from the fit, gives $A_1 \approx 0.1 \cdot 10^4\ \text{cm}^{-2}$, this value being even lower than that for the BHA free protein.

The effective constant of the electron-vibrational interaction, B_1, is approximately two times reduced by substrate binding, the effective frequency being weakly affected. It follows from this result and Eq. (6) that BHA binding reduces the number or amplitude of vibrations contributing to the band broadening.

There is a weak deviation of the experimental values from the theoretical curve at $T > T_c$. This implies that the $Q(0, 0)$ absorption band is coupled mainly to the heme vibrations, which are not affected by the glass-liquid phase transition. This conclusion is supported by the fact that temperature dependence of M_1 (Fig. 4 *A*) of both compounds is notable already in the glassy state (~140–150 K), and its character is not affected by the phase transition at ~170–180 K.

Henriksen et al. (1998) showed that there are nonbonded interactions between BHA and part of the heme periphery. These contacts restrain the mobility of this part of the heme; the higher the temperature, the stronger the restriction. Consequently, the BHA binding is expected to diminish the increase of the amplitude of the corresponding vibrations upon heating. Assuming that these vibrations are coupled to the $Q(0, 0)$ transition, one comes to the conclusion that the BHA binding is expected to reduce the temperature broadening of this band. Because this part of the heme is not exposed to the solvent, its vibrations are not expected to depend strongly on the solvent phase transition. This conclusion corroborates with the presented above results that the BHA binding reduces the temperature broadening of the $Q(0, 0)$, whereas the solvent glass-liquid phase transition weakly affects it.

IR CO absorption band

In the case of the CO IR absorption band B_i is a constant of the linear anharmonic coupling of the CO vibration to some other *i*-th vibration. It can be a vibration of the heme or its environment (protein amino acids or solvent molecules). In the latter case the heme environment motion affects the IR transition through the dynamically changing protein or/and solvent electric fields. Interpretation of the experimental data on the temperature dependence of M_2 of the CO IR absorption band of the HRP(CO) and Mb(CO) complexes showed that it is controlled by the anharmonic coupling with the heme environment. Therefore, M_2, of the CO IR absorption band of heme protein, provides information on dynamics of the heme environment and its temperature dependence; contribution of the coupling with other heme modes hardly affects the temperature dependence under consideration.

It has been shown experimentally (Park et al., 1991; Ray et al., 1994; Laberge et al., 1996; Kaposi et al., 1999; Phillips et al., 1999) that the CO frequency is strongly

affected by the electric field produced by the heme environment. The magnitude of the effect of the heme environment can be estimated based on results by Kushkuley and Stavrov (1996, 1997). It follows from these works that a unit charge can considerably affect ν(CO) only if amplitude of this motion is of the order of 1 Å. Moreover, even large amplitude (~1 Å) motion of a charged particle can essentially affect ν(CO) only if this particle is located not far from the heme. Therefore, only large amplitude (and low-frequency, as a result) motions of the atomic groups located close to the heme can considerably contribute to the CO band broadening.

Interpreting the experimentally observed M_2 temperature dependence of the HRP(CO) and Mb(CO) complexes, we assumed that the solvent glass-liquid phase transition strongly affects dynamics of the heme environment. This assumption is supported by the results of all experimental techniques used to study behavior of this protein: kinetics of carbon monoxide rebinding (Austin et al., 1975; Ansari et al., 1987; Steinbach et al., 1991; Post et al., 1993), x-ray diffraction (Frauenfelder et al., 1979; Hartmann et al., 1982; Parak and Knapp, 1984), Mössbauer spectroscopy (Dvivedi et al., 1979; Keller and Debrunner, 1980; Parak et al., 1982; Mayo et al., 1983; Parak and Knapp, 1984; Frauenfelder et al., 1988; Nienhaus et al., 1992a; Melchers et al., 1996), inelastic neutron (Doster et al., 1989; Cusack and Doster, 1990) and x-ray scattering (Achterhold et al., 1996), optical absorption spectroscopy in the visible and close ultraviolet intervals (Cordone et al., 1986, 1988; Di Iorio et al., 1991; Di Pace et al., 1992; Nienhaus et al., 1992; Cupane et al., 1993; Boffi et al., 1994), and pressure effects on IR absorption (Frauenfelder et al., 1990), and resonance Raman (Galkin et al., 1997) spectra. Melchers et al. (1996) showed that changes in the optical absorption Soret band stem mainly from changes in the structure and/or dynamics of the heme group and its closest environment. All these results show that the phase transition of the solvent induces changes in the structure and dynamics of the whole protein and, in particular, in electronic and vibrational structure of the heme-imidazole unit. The results of Doster et al. (1987) on kinetics of carbon monoxide recombination with HRP show that dynamics of this protein is also affected by the glass-liquid phase transition.

Taking into account the effect of the glass-liquid phase transition allowed to explain the experimentally observed temperature dependencies of the HRP(CO)-BHA CO band and Mb(CO) A_1 band as a result of the anharmonic coupling of the CO vibration with motion of the heme environment (Fig. 6 *B*).

The steep temperature dependencies of the HRP(CO) band and Mb(CO) A_0 band (Fig. 6 *B*) indicate that motion of the heme environment is strongly anharmonic. Following Doster et al. (1989), we assumed that an excited state is located close to the ground one. In this approach the experimentally observed temperature dependencies were successfully fitted, parameters of these two states being presented in Table 2. It follows from the results that elastic constants of the low-frequency motions of the heme environment are much smaller and/or number of these vibrations is much larger in the excited substate.

The very different properties of the ground and excited states can be caused by the very different network of hydrogen bonds or specific electrostatic interactions in these substates. In the ground substate this network strongly increases the elastic constant and reduces the amplitude of the heme environment motion, which contributes to the temperature dependence under consideration. Consequently, these motions weakly contribute to the CO band temperature broadening. In the excited state this network is broken, at least partially. The active motions of the heme environment become of large amplitude and of low frequency, and, consequently, strongly contribute to the M_2 temperature dependence. The presence of two close protein states (conformational substrates) with very different elastic properties along some vibrational coordinate(s) causes strongly anharmonic character of the heme environment motion along these coordinates.

Binding of substrate by HRP(CO) affects the protein structure and dynamics in two ways (Henriksen et al., 1998). First, it forms a number of hydrogen bonds with amino acids of the heme pocket, including H42, R38, and P139. Second, the easy access of solvent molecules to the heme group is hindered by the motion of F68, which twists to close the aqueous pore. Formation of hydrogen bonds with the number of amino acids anchors their large-amplitude motions, making the heme pocket more rigid (note that, in general, the substrate binding does not necessarily make protein more rigid, e.g., Gunsalus et al. (1977)). As a result, the conformational substate, corresponding to the presence of large amplitude motions, has much higher energy and does not become populated even at room temperatures. Hindrance of the solvent access reduces the number of the solvent molecules located in the heme pocket comparing to the free substrate enzyme (Gajhede et al., 1997). Contribution of the solvent molecules' motion to the CO broadening is also reduced. Combination of these two effects explains strong difference between the temperature behavior of the IR absorption spectra of these compounds.

From the same point of view, the difference between the temperature behaviors of the A_0 and A_1 bands of Mb(CO) can be understood (Fig. 6 *B*). It was shown (Morikis et al., 1989; Zhu et al., 1992; Yang and Phillips, 1996; Kushkuley and Stavrov, 1997; Müller et al., 1999) that A_0 band corresponds to the "open" protein conformation with the distal histidine located out of the heme pocket, whereas the A_1 band corresponds to the "closed" conformation with the distal histidine located in the pocket. In the open conformation there is much more free space in the heme pocket and, consequently, it can accommodate solvent molecules even in the presence of the coordinated CO molecule. Moreover,

in this conformation a number of hydrogen bond are broken (in particular, the hydrogen bond between the heme propionate group and Arg45), making the protein more flexible. These structural differences cause much stronger temperature dependence of the A_0 band compared with the A_1 band.

One could assume that the temperature broadening under consideration could be explained only invoking into consideration effect of the solvent molecules located in the heme pocket. In this case the ground state could correspond to a state with solvent molecules bonded to an atom or group of atoms forming the pocket, whereas the excited state could correspond to a state where the solvent molecule moves inside the pocket. This mechanism could explain the high temperature CO band narrowing upon the substrate binding to HRP(CO) or presence of the distal histidine in the pocket of Mb(CO) as a result of a displacement of the solvent molecules from the heme pocket. However, this assumption does not explain the CO bandwidth independence of temperature in the glassy state. Indeed, the phase transition affects the protein dynamics and does not affect directly binding of the solvent molecules inside the heme pocket. Therefore, one must assume that the ground and excited states under consideration correspond to different conformational substates of the protein.

Note also that the low-temperature magnitudes of M_2 of the IR CO absorption bands of HRP(CO) and HRP(CO)-BHA are very close, being in the framework of the experimental error. The difference between the lowest temperature second moments of the corresponding $Q(0, 0)$ bands (taking into account the band splitting in HRP(CO)-BHA) is also small. The effects of the substrate binding to HRP(CO) on the width of the bands under consideration becomes notable at higher temperatures. This result nicely corroborates with finding of Balog et al. (1997, 2000) that the substrate binding weakly affects the compressibility of Mg-mesoporphyrin-HRP at 1.7 K.

CONCLUSION

We considered how the protein globule modulates the function of the heme, and whether the viscosity of the exterior solvent influences the polypeptide chain such that the heme physical characteristics are changed. Two spectroscopic markers were used, namely IR and visible absorption. Temperature dependence of these markers depends on different kinds of interactions.

It was shown that broadening of the $Q(0, 0)$ band of HRP(CO) and HRP(CO)-BHA mainly stems from electron-vibrational interaction with the heme vibrations, the interaction with protein motions being considerably weaker. Therefore, the width of this band is weakly affected by the glass-liquid phase transition of the solvent.

In contrast, the contribution of the anharmonic coupling of the CO vibration with the heme vibrations hardly contributes to the CO IR absorption band. Therefore, broadening of this band stems mainly from the anharmonic coupling with large amplitude motions of the heme pocket amino acids and solvent molecules, if such motions are present. As a result, constancy of the CO band shape of HRP(CO) and Mb(CO) below temperature of the glass-liquid phase transition and its strong temperature dependence at higher temperatures reflect very different properties of the protein in the glassy and liquid states of the solvent. In the glassy state, motions of the heme pocket amino acids are frozen in into the solvent matrix and the width remains constant, whereas, in the liquid state, amplitudes of these motions increase upon heating and increase the bandwidth.

The very steep broadening of the CO band of HRP(CO) and A_0 band of Mb(CO) upon heating were interpreted as a manifestation of the presence of an excited conformational substate located close to the ground one. This excited substate is characterized by the presence of large amplitude vibrations of the heme pocket amino acids (and, probably, solvent molecules located in the pocket), whereas in the ground state these vibrations have considerably higher elastic constants. This difference is caused most probably by very different network of hydrogen bonds or specific electrostatic interactions in these substates. In the ground substate this network strongly increases the elastic constant and reduces the amplitude of the heme pocket motions. In the excited substate this network is broken, at least partially, the corresponding elastic constants are considerably reduced and the vibrations have large amplitude.

The substrate analog BHA forms a number of hydrogen bonds with the heme pocket amino acids. These new bonds "anchor" the amino acids, causing sharp increases in the elastic constants of their motions. The BHA binding may also hinder access of water into the pocket. As a result, amplitudes of the motions under consideration are much smaller and the interaction with them much weaker contributes to the CO band broadening. Therefore, the temperature dependence of the CO band of HRP(CO)-BHA is much weaker than that of HRP(CO). The substrate binding also significantly weakens the temperature dependence of the $Q(0, 0)$ band, this weakening manifests strong reduction of amplitudes of heme vibrations interacting with the $S_0 \rightarrow S_1$ electronic transition. The conclusion that the optical and IR absorption spectra reflect interactions with different vibrations is supported by the fact that the latter are very sensitive to the glass-liquid phase transition of the solvent, whereas the former are weakly affected by the phase transition.

It follows from our study that presence of the low-energy flexible excited conformational substate is not an exclusive property of HRP; it presents in other proteins also, e.g., in myoglobin. It is possible that this substate is functionally important, being responsible for the penetration of different ligands into the heme pocket and their accommodation there.

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REFERENCES

Achterhold, K., C. Keppler, U. van Bürk, W. Potzel, P. Schindelmann, E. W. Knapp, B. Melchers, A. I. Chumakov, A. Q. Baron, R. Rüffer, and F. Parak. 1996. Temperature dependent inelastic X-ray scattering of synchrotron radiation of myoglobin analyzed by the Mössbauer effect. *Eur. Biophys. J.* 25:43–46.

Anderson, W. P., W. D. Edwards, and M. C. Zerner. 1986. Calculated spectra of hydrated ions of the first transition-metal series. *Inorg. Chem.* 25:2728–2732.

Ansari, A., J. Berendzen, D. Braunstein, B. R. Cowen, H. Frauenfelder, M. K. Hong, I. E. Iben, J. B. Johnson, P. Ormos, T. B. Sauke, R. Scholl, A. Schulte, P. J. Steinbach, J. Vittitow, and R. D. Young. 1987. Rebinding and relaxation in the myoglobin pocket. *Biophys. Chem.* 26: 337–355.

Austin, R. H., K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus. 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* 14:5355–5373.

Bacon, A. D., and M. C. Zerner. 1979. An intermediate neglect of differential overlap theory for transition metal complexes: Fe, Co and Cu chlorides. *Theor. Chim. Acta.* 53:21–54.

Balog, E., K. Kis-Petik, J. Fidy, M. Kohler, and J. Friedrich. 1997. Interpretation of multiple Q(0,0) bands in the absorption spectrum of Mg-mesoporphyrin embedded in horseradish peroxidase. *Biophys. J.* 73:397–405.

Balog, E., R. Galantai, M. Köhler, M. Laberge, and J. Fidy. 2000. Metal coordination influences substrate binding in horseradish peroxidase. *Eur. Biophys. J.* 29:429–438.

Barlow, C. H., P. I. Ohlsson, and K. G. Paul. 1976. Infrared spectroscopic studies of carbonyl horseradish peroxidases. Biochemistry. 15: 2225–2229.

Bitler, A., and S. S. Stavrov. 1999. Iron-histidine resonance Raman band of deoxyheme proteins: effects of anharmonic coupling and glass-liquid phase transition. *Biophys. J.* 77:2764–2776.

Berinstain, A. B., A. M. English, B. C. Hill, and D. Sharma. 1990. Picosecond flash photolysis of carboxy horseradish peroxidase: rapid geminate recombination in the presence of benzohydroxamic acid. *J. Am. Chem. Soc.* 112:9649–9651.

Boffi, A., D. Verzilli, E. Chiancone, M. Leone, A. Cupane, V. Militello, E. Vitrano, L. Cordone, W. Yu, and E. E. Di Iorio. 1994. Stereodynamic properties of the cooperative homodimeric Scapharca inaequivalvis hemoglobin studied through optical absorption spectroscopy and ligand rebinding kinetics. *Biophys. J.* 67:1713–1723.

Cordone, L., A. Cupane, M. Leone, and E. Vitrano. 1986. Optical absorption spectra of deoxy- and oxyhemoglobin in the temperature range 300–20 K. Relation with protein dynamics. *Biophys. Chem.* 24: 259–275.

Cordone, L., A. Cupane, M. Leone, and E. Vitrano. 1988. Interaction between external medium and haem pocket in myoglobin probed by low-temperature optical spectroscopy.1988. *J. Mol. Biol.* 199:213–218.

Cupane, A., M. Leone, and E. Vitrano. 1993a. Protein dynamics: conformational disorder, vibrational coupling and anharmonicity in deoxyhemoglobin and myoglobin. *Eur Biophys. J.* 21:385–391.

Cupane, A., M. Leone, E. Vitrano, and L. Cordone. 1988. Structural and dynamic properties of the heme pocket in myoglobin probed by optical spectroscopy. *Biopolymers.* 27:1977–1997.

Cupane, A., M. Leone, E. Vitrano, L. Cordone, U. R. Hiltpold, K. H. Winterhalter, W. Yu, and E. E. Di Iorio. 1993b. Structure-dynamics-function relationships in Asian elephant (*Elephas maximus*) myoglobin. An optical spectroscopy and flash photolysis study on functionally important motions. *Biophys. J.* 65:2461–2472.

Cusack, S., and W. Doster. 1990. Temperature dependence of the low-frequency dynamics of myoglobin. Measurement of the vibrational frequency distribution by inelastic neutron scattering. *Biophys. J.* 58: 243–251.

Di Iorio, E. E., U. R. Hiltpold, D. Filipovic, H. Winterhalter, E. Gratton, E. Vitrano, A. Cupane, M. Leone, and L. Cordone. 1991. Protein dynamics. Comparative investigation on heme-proteins with different physiological roles. *Biophys. J.* 59:742–754.

Di Pace, A., A. Cupane, M. Leone, E. Vitrano, and L. Cordone. 1992. Protein dynamics. Vibrational coupling, spectral broadening mechanisms, and anharmonicity effects in carbonmonoxy heme proteins studied by the temperature dependence of the Soret band lineshape. *Biophys. J.* 63:475–484.

Doster, W., S. Cusack, and W. Petry. 1989. Dynamical transition of myoglobin revealed by inelastic neutron scattering. *Nature.* 337: 754–756.

Doster, W., S. F. Bowne, H. Frauenfelder, L. Reinisch, and E. Shyamsunder. 1987. Recombination of carbon monoxide to ferrous horseradish peroxidase types A and C. *J. Mol. Biol.* 194:299–312.

Du, P., F. U. Axe, G. H. Loew, S. Canuto, and M. C. Zerner. 1991. Theoretical study on the electronic spectra of model compound II complexes of peroxidases. *J. Amer. Chem. Soc.* 113:8614–8621.

Dvivedi, A., T. Pederson, and P. G. Debrunner. 1979. Recoilless fraction of iron proteins in frozen solution. *J. Physique C2.* 40:C2–531–C2–533.

Edwards, W. D., B. Weiner, and M. C. Zerner. 204. 1986. On the low-lying states and electronic spectroscopy of iron(II) porphine. *J. Amer. Chem. Soc.* 108:2196–2202.

Evangelista-Kirkup, R., G. Smulevich, and T. G. Spiro. 1986. Alternative carbon monoxide binding modes for horseradish peroxidase studied by resonance Raman spectroscopy. *Biochemistry.* 25:4420–4425.

Frauenfelder, H., F. Parak, and R. D. Young. 1988. Conformational substrates in proteins. *Annu. Rev. Biophys. Biochem.* 17:451–479.

Frauenfelder, H., G. A. Petsko, and D. Tsernoglu. 1979. Temperature-dependent X-ray diffraction as a probe of protein structural dynamics. *Nature.* 280:558–563.

Frauenfelder, H., G. U. Nienhaus, and R. D. Young. 1994. Relaxation and disorders in proteins. *In* Richert R., Blumen J., editors. Disorder Effects on Relaxational Processes. Berlin, Springer-Verlag. 592–614.

Frauenfelder, H., N. A. Alberding, A. Ansari, D. Braunstein, B. R. Cowen, M. K. Hong, I. E. T. Iben, J. B. Johnson, S. Luck, M. C. Marden, J. R. Mourant, P. Ormos, L. Reinisch, R. Scholl, A. Schulte, E. Shyamsunder, L. B. Sorensen, P. J. Steinbach, A. Xie, R. D. Young, and K. T. Yue. 1990. Proteins and pressure. *J. Phys. Chem. B.* 94:1024–1037.

Gajhede, M., D. J. Schuller, A. Henriksen, A. T. Smith, and T. L. Poulos. 1997. Crystal structure of horseradish peroxidase C at 2.15 A resolution. *Nat. Struct. Biol.* 4:1032–1038.

Galkin, O., S. Buchter, A. Tabirian, and A. Schulte. 1997. Pressure effects on the proximal heme pocket in myoglobin probed by Raman and near-infrared absorption spectroscopy. *Biophys. J.* 73:2752–2763.

Gouterman, M. 1978. Optical spectra and electronic structure of porphyrins and related rings. *In* Dolphin D., editor. The Porphyrins. New York, Academic Press. 1–156.

Gunsalus, I. C., S. G. Sligar, T. Nordlund, and H. Frauenfelder. 1977. Oxygen sensing heme proteins: monooxygenases, myoglobin and hemoglobin. *Adv. Exp. Med. Biol.* 78:37–50.

Hartmann, H., F. Parak, W. Stegemann, G. A. Petsko, D. Ringe Ponzi, and H. Frauenfelder. 1982. Conformational substrates in a protein: structure and dynamics of metmyoglobin at 80 K. *Proc. Natl. Acad. Sci. U.S.A.* 79:4967–4971.

Hartmann, H., S. Zinser, P. Komninos, G. U. Nienhaus, and F. Parak. 1996. X-ray structure determination of a metastable state of carbonmonoxy myoglobin after photodissociation. *Proc. Natl. Acad. Sci. U.S.A.* 93: 7013–7016.

Henriksen, A., D. J. Schuller, K. Meno, K. G. Welinder, A. T. Smith, and M. Gajhede. 1998. Structural interactions between horseradish peroxidase C and the substrate benzhydroxamic acid determined by X-ray crystallography. *Biochemistry.* 37:8054–8060.

Jeffrey, G. A. 1997. An Introduction to Hydrogen Bonding. New York, Oxford University Press.

Kaposi, A. D., J. Fidy, E. S. Manas, J. M. Vanderkooi, and W. W. Wright. 1999. Horseradish peroxidase monitored by infrared spectroscopy: Effect of temperature, substrate and calcium. *Biochim. Biophys. Acta.* 1435:41–50.

Kaposi, A. D., W. W. Wright, J. Fidy, S. S. Stavrov, J. M. Vanderkooi, and I. Rasnik. 2001. Carbonmonoxy horseradish peroxidase as a function of pH and substrate: Influence of local electric fields on the optical and infrared spectra. *Biochemistry.* 40:3483–3491.

Keller, H., and P. G. Debrunner. 1980. Evidence for conformational and diffusional mean square displacements in frozen aqueous solution of oxymyoglobin. *Phys. Rev. Lett.* 45:68–71.

Kushkuley, B., and S. S. Stavrov. 1996. Theoretical study of the distal-side steric and electrostatic effects on the vibrational characteristics of the FeCO unit of the carbonylheme proteins and their models. *Biophys. J.* 70:1214–1229.

Kushkuley, B., and S. S. Stavrov. 1997. Theoretical study of the electrostatic and steric effects on the spectroscopic characteristics of the metal-ligand unit of heme proteins. 2. C-O vibrational frequencies, ^{17}O isotropic chemical shifts and nuclear quadruple coupling constants. *Biophys. J.* 72:899–912.

Laberge, M., J. M. Vanderkooi, and K. A. Sharp. 1996. Effect of a protein electric field on the CO-stretch frequency. Finite difference Poisson-Boltzmann calculations on carbonmonoxy cytochromes *c*. *J. Phys. Chem. B.* 100:10793–10801.

Laberge, M., M. Köhler, J. M. Vanderkooi, and J. Friedrich. 1999. Sampling field heterogeneity at the heme of *c*-type cytochromes by spectral hole burning and electrostatic calculations. *Biophys. J.* 77:3293–3304.

Lax, M. 1952. The Franck-Condon principle and its application to crystals. *J. Chem. Phys.* 20:1752–1760.

Manas, E., J. M. Vanderkooi, and K. Sharp. 1999. The effects of protein environment on the low temperature electronic spectra of cytochrome *c* and microperoxidase. *J. Phys. Chem. B.* 103:6334–6348.

Markham, J. J. 1959. Interaction of normal modes with electron traps. *Rev. Mod. Phys.* 31:956–989.

Mayo, K. H., D. Kucheida, F. Parak, and J. C. Chien. 1983. Structural dynamics of human deoxyhemoglobin and hemochrome investigated by nuclear γ resonance absorption (Mössbauer) spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* 80:5294–5296.

Melchers, B., E. W. Knapp, F. Parak, L. Cordone, A. Cupane, and M. Leone. 1996. Structural fluctuations of myoglobin from normal-modes, Mössbauer, Raman, and absorption spectroscopy. *Biophys. J.* 70: 2092–2099.

Morikis, D., P. M. Champion, B. A. Springer, and S. G. Sligar. 1989. Resonance Raman investigations of site-directed mutants of myoglobin: effects of distal histidine replacement. *Biochemistry.* 28:4791–4800.

Müller, J. D., B. H. McMahon, E. Y. Chien, S. G. Sligar, and G. U. Nienhaus. 1999. Connection between the taxonomic substrates and protonation of histidines 64 and 97 in carbonmonoxy myoglobin. *Biophys. J.* 77:1036–1051.

Nienhaus, G. U., J. R. Mourant, and H. Frauenfelder. 1992b. Spectroscopic evidence for conformational relaxation in myoglobin. *Proc. Natl. Acad. Sci. U.S.A.* 89:2902–2906.

Nienhaus, G. U., H. Frauenfelder, and F. Parak. 1992a. Structural fluctuations in glass-forming liquids: Mössbauer spectroscopy on iron in glycerol. *Phys. Rev. B.* 3:3345–3350.

Nienhaus, G. U., J. R. Mourant, K. Chu, and H. Frauenfelder. 1994. Ligand binding to heme proteins: the effect of light on ligand binding in myoglobin. *Biochemistry.* 33:13413–13430.

Parak, F., and E. W. Knapp. 1984. A consistent picture of protein dynamics. *Proc. Natl. Acad. Sci. U.S.A.* 81:7088–7092.

Parak, F., E. W. Knapp, and D. Kucheida. 1982. Protein dynamics. Mössbauer spectroscopy on deoxymyoglobin crystals. *J. Mol. Biol.* 161: 177–194.

Park, K. D., K. Guo, F. Adebodun, M. L. Chiu, S. G. Sligar, and E. Oldfield. 1991. Distal and proximal ligand interactions in heme proteins: correlations between C-O and Fe-C vibrational frequencies, oxygen-17 and carbon-13 nuclear magnetic resonance. *Biochemistry.* 30: 2333–2347.

Paul, K.-G. 1958. Die Isolierung von Meerrettichperoxidase. *Acta Chem. Scand.* 12:1312–1318.

Perlin, Yu. E. 1964. Modern methods in the theory of many-phonon processes. *Usp. Fiz. Nauk.* 80:553–595.

Phillips, G. N. Jr., M. L. Teodoro, T. Li, B. Smith, and J. S. Olson. 1999. Bound CO is a molecular probe of electrostatic potential in the distal pocket of myoglobin. *J. Phys. Chem. B.* 103:8817–8829.

Post, F., W. Doster, G. Karvounis, and M. Settles. 1993. Structural relaxation and nonexponential kinetics of CO-binding to horse myoglobin: multiple flash photolysis experiments. *Biophys. J.* 64:1833–1842.

Ray, G. B., X. Y. Li, J. A. Ibers, J. L. Sessler, and T. G. Spiro. 1994. How far can proteins bend the FeCO unit? Distal polar and steric effects in heme proteins and models. *J. Amer. Chem. Soc.* 116:162–176.

Rebane, K. K. 1970. Impurity Spectra of Solids. Elementary Theory of Vibrational Structure. New York, Plenum Press.

Rosenfeld, Yu. B., and S. S. Stavrov. 1994. Anharmonic coupling of soft modes and its influence on the shape of the iron-histidine resonance Raman band of heme proteins. *Chem. Phys.* 229:457–464.

Sanfratello, V., A. Boffi, A. Cupane, and M. Leone. 2000. Heme symmetry, vibronic structure, and dynamics in heme proteins: ferrous nicotinate horse myoglobin and soybean leghemoglobin. *Biopolymers.* 57: 291–305.

Smulevich, G., R. Evangelista-Kirkup, A. English, and T. G. Spiro. 1986. Raman and infrared spectra of cytochrome *c* peroxidase-carbon monoxide adducts in alternative conformational states. *Biochemistry.* 25: 4426–4430.

Stavrov, S. S., I. P. Decusar, and I. B. Bersuker. 1993. Chemical activation of oxygen and carbon monoxide by hemoproteins: the vibronic approach. *New J. Chem.* 17:71–76.

Steinbach, P. J., A. Ansari, J. Berendzen, D. Braunstein, K. Chu, B. R. Cowen, D. Ehrenstein, H. Frauenfelder, J. B. Johnson, D. C. Lamb, C. Luck, J. R. Mourant, G. U. Nienhaus, P. Ormos, R. Philipp, A. Xie, and R. D. Young. 1991. Ligand binding to heme proteins: connection between dynamics and function. *Biochemistry.* 30:3988–4001.

Tamura, M., T. Asakura, and T. Yonetani. 1972. Heme-modification studies on horseradish peroxidase. *Biochim. Biophys. Acta.* 268: 292–304.

Uno, T., Y. Nishimura, M. Tsuboi, R. Makino, T. Iizuka, and Y. Ishimura. 1987. Two types of conformers with distinct Fe-C-O configuration in the ferrous CO complex of horseradish peroxidase. Resonance Raman and infrared spectroscopic studies with native and deuteroheme-substituted enzymes. *J. Biol. Chem.* 262:4549–4556.

Yang, F., and G. N. Phillips Jr. 1996. Crystal structure of CO-, deoxy- and met-myoglobins at various pH values. *J. Mol. Biol.* 256:762–774.

Zhu, L., J. T. Sage, A. A. Rigos, D. Morikis, and P. M. Champion. 1992. Conformational interconversion in protein crystals. *J. Mol. Biol.* 224: 207–215.