# The Enzyme Horseradish Peroxidase Is Less Compressible at Higher Pressures

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ABSTRACT Fluorescence line-narrowing (FLN) spectroscopy at 10 K was used to study the effect of high pressure through the prosthetic group in horseradish peroxidase (HRP), which was Mg-mesoporphyrin (MgMP) replacing the heme of the enzyme. The same measurement was performed on MgMP in a solid-state amorphous organic matrix, dimethyl sulfoxide (DMSO). Series of FLN spectra were registered to determine the (0, 0) band shape through the inhomogeneous distribution function (IDF). In the range of 0–2 GPa a red-shift of the IDF was determined, and yielded the isothermal compressibility of MgMP-HRP as 0.066 $GPa^{-1}$, which is significantly smaller than that found earlier as 0.106 $GPa^{-1}$ by fine-tuning the pressure in the range up to 1.1 MPa. The vibrational frequencies also shifted with pressure increase, as expected. The compressibility in the DMSO matrix was smaller, 0.042 $GPa^{-1}$, both when the pressure was applied at room temperature before cooling to 10 K, or at 10 K. At 200 K or above, the bimodal (0, 0) band shape in DMSO showed a population conversion under pressure that was not observed at or below 150 K. A significant atomic rearrangement was estimated from the volume change, 3.3 $\pm$ 0.7 $cm^3$/mol upon conversion. The compressibility in proteins and in amorphous solids seems not to significantly depend on the temperature and in the protein it decreases toward higher pressures.

## INTRODUCTION

Heme proteins play an essential role in numerous important biochemical processes, e.g., in $O_2$ transport and storage, in electron transfer, and in the catalysis of peroxidative reactions. The biochemistry of the heme group is the key issue in the understanding of these phenomena. The heme, however, is not only a very important prosthetic group, but because of its spectral properties it offers a unique way of monitoring for biophysical techniques, such as resonance Raman, optical absorption, and fluorescence spectroscopy (Spiro, 1988; Kincaid, 2000; Gouterman, 1978; Cupane et al., 1995; Fidy et al., 1998a). These techniques, which are based on the properties of the vibronic manifold of the optical marker, provide information about the environment in the protein through the perturbation of the electronic or vibrational states. In the case of hemoproteins such studies are of special interest because the tested environment is the active site of the protein.

Pressure is a thermodynamic parameter, which is equally important, but not as widely used like the temperature. Pressure-dependent studies, however, are also able to yield important thermodynamic parameters of the system (Silva and Weber, 1993; Heremans, 1997; Heremans and Smeller, 1998). Studies by high-resolution optical spectroscopy have shown that the electronic excitation energy is sensitive to pressure changes in the low pressure range up to 1–2 MPa, [pressure units: 1 GPa = 10 kbar; 0.1 GPa = 100 MPa = 1 kbar; 0.1 MPa = 1 bar = atmospheric pressure] inducing fully reversible effects (Zollfrank et al., 1991; Fidy et al., 1998a). Higher but moderate pressures (0.1–0.5 GPa) distort the secondary structure still in a reversible way. (Heremans et al., 1996; Ruan et al., 1999; Kunugi et al., 1997; Mozhaev et al., 1996; Heinisch et al., 1995; Jonas and Jonas, 1994; Jonas et al., 1998; Freiberg et al., 1993; Frauenfelder et al., 1990; Sturgis et al., 1998). This pressure range is particularly interesting because it affects the intermolecular interactions (Silva and Weber, 1988; Smeller et al., 1999). Pressures in the 0.5–1 GPa range can cause protein denaturation (Panick et al., 1999; Gross and Jaenicke, 1994; Zhang et al., 1995; Balny et al., 1992; Hayashi and Balny, 1996; Heremans and Smeller, 1998), thus this range is critical in determining the denaturation phase diagram and pressure stability of proteins. In this work, the combination of high hydrostatic pressure with low temperature kept the protein samples always in the reversible range, because conformational changes that could be irreversible need extensive rearrangement of the protein, which is not possible (or very slow) at cryogenic temperatures.

A unique possibility of pressure-dependent optical spectroscopy is that the compressibility of the protein can be determined as experienced by an embedded chromophore. Zollfrank et al. (1991) determined the intrinsic compressibility of a protein for the first time using spectral hole-burning spectroscopy at cryogenic temperatures. These studies were followed by a series of works on proteins by the same technique (for a review see Fidy et al., 1998b). Protein compressibility can also be determined by ultrasound velocity measurements (Kharakoz and Sarvazyan, 1993; Chalikian and Breslauer, 1998) around room temperature. This method was used by Gekko for the systematic survey of a large number of proteins (Gekko, 1991; Tamura and Gekko, 1995). One has to note, however, that besides

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the temperature difference, the two techniques lead to physically different parameters. Optical spectroscopy yields the isothermal compressibility of the interacting environment of a chromophore, while by ultrasound velocity one is able to determine the adiabatic bulk compressibility of the whole solution. From these data the protein compressibility can only be obtained if one corrects for the contribution of the solvent. One needs to perform calculations that involve the knowledge of such parameters as the heat capacity ($c_p$) and the thermal expansion coefficient of the protein to obtain the isothermal compressibility as a bulk protein-parameter (Gekko and Noguchi, 1979). In this work we have used fluorescence line-narrowing (FLN) spectroscopy, a high-resolution technique of laser spectroscopy (Personov, 1983).

This technique was combined with the application of high pressure. Spectroscopy was performed on an embedded fluorescent porphyrin molecule Mg-mesoporphyrin (MgMP), replacing the heme at the active site of an enzyme, horseradish peroxidase (HRP). The laser excitation of fluorescence in FLN allowed us to collect enough signal even from a sample volume as small as in a diamond anvil cell that we used to generate high pressure up to 2.1 GPa. The FLN method made it possible to determine the true inhomogeneous band of the electronic origin (free of temperature broadening and phonon coupling) with high precision at varying pressures. The band position and width allowed detection of the pressure-induced shift and broadening of the inhomogeneous (0, 0) band, and thus to determine the isothermal compressibility of the protein.

Although there are spectral hole-burning studies reported in the literature that were performed under high pressure (Koedijk et al., 1995; Lock et al., 1999; Laisaar and Kikas, 1998; Kikas et al., 1998; Reddy et al., 1996; Wu et al., 2000) there were no high-pressure FLN measurements reported on biological systems. We show in this paper that the FLN method is applicable to reveal the pressure-induced perturbation of the protein matrix monitored at the active site of the enzyme.

According to the fluctuation-dissipation theorem of statistical physics (Landau and Lifsitz, 1969; Cooper, 1976) the volume fluctuations $\langle V - \langle V\rangle\rangle$ of a system arising from conformational dynamics can be characterized by its compressibility:

$$\langle V - \langle V\rangle\rangle = \sqrt{\kappa k_B T \langle V\rangle},$$

where $V$ is the volume, $\kappa$ the compressibility, $k_B$ the Boltzmann constant, $T$ the temperature, and angle brackets indicate statistical average. Because the volume fluctuations are proportional to the square root of the compressibility, pressure-dependent experiments can reveal important aspects of the biological processes even if the pressures applied in these measurements are beyond the values observed in the biosphere. It is becoming better understood that besides the biochemical properties, specific conformational dynamics/mobility are also requirements for biological macromolecular systems such as proteins to fulfill their biological function.

The native heme group in hemoproteins is not suitable for high-resolution optical spectroscopy because the excited-state lifetime of Fe-protoporphyrin is very short, which leads to inherently broad spectral lines. The usual method to overcome this problem is the substitution of the heme by a fluorescent porphyrin to make the sample appropriate for either hole-burning spectroscopy or for FLN studies. In the present studies we have been using MgMP substitution for the heme in HRP (Balog et al., 1997). Previous studies in our laboratory have shown that the association constant of substrate molecules of the enzyme binding in the heme pocket was somewhat decreased in these samples, but still was high (250 $mM^{-1}$ for naphtohydroxamic acid (Balog et al., 2000)). As this substrate binds in the vicinity of the heme, this result showed that after the substitution, the protein structure did not become significantly disturbed, not even around the heme site.

## MATERIALS AND METHODS

### Sample preparations

MgMP was purchased from Porphyrin Products (Logan, UT). For the studies performed in the protein mimic glassy matrix, MgMP was dissolved in dimethylsulfoxide (DMSO) at a concentration of 7.5 mM. For the protein studies HRP was purchased from Sigma (St. Louis, MO) RZ = 3.0). To purify the basic form (isoenzyme C) of this protein, column chromatography was used (Paul, 1958). The protein was applied to a CM Sepharose (Pharmacia, Piscataway, NJ) in a column equilibrated with 5 mM Tris buffer at pH 7.4 and eluted with 0.01 M NaCl. The RZ values of the fractions were checked by a Cary 4E (Varian) absorption spectrophotometer, and the fraction RZ = 3.4 was used for the heme substitution. The native heme group was removed from the enzyme by acid methyl ethyl ketone (Teale, 1959) and the apoprotein was reconstituted with aliquots of MgMP dissolved in ethanol. Samples of MgMP-HRP were prepared at a concentration of 1.6 mM. For the experiment, a 10 mM pH 7 phosphate buffer containing 50% glycerol was used to ensure transparency of the sample.

### Experimental methods

The exciting light source was a CW ring dye laser (Coherent 899-01, Coherent Inc., Santa Clara, CA) pumped by an argon ion laser (Coherent Innova 307). The sample was pressurized in a special low-temperature diamond anvil cell (DAC) (Diacell Products, Leicester, UK). This cell of ~40 nl volume allows us to apply pressures in the range of 0–2 GPa. The DAC was mounted on the cold finger of a closed cycle helium cryostat (Cryophysics, Geneva, Switzerland) that cooled the sample to 10 K. A helium gas-driven membrane system allowed us to change the pressure in the whole temperature range. The temperature was measured by silicon diodes on the body of the DAC. A ruby chip was used for the pressure determination (Forman et al., 1972), which also made it possible to check the temperature inside the cell (Weinstein, 1986). The accuracy of the pressure values measured at low temperatures was typically 10 MPa (Smeller, 1999). The pressure measurements at the beginning and end of the measurement did not differ >30 MPa. The spectra were recorded by a

Jobin-Yvon monochromator equipped with a cooled photomultiplier operating in photon counting mode (Hamamatsu).

## FLN experiments

The FLN experiment requires cryogenic temperatures and excitation by laser light of narrow linewidth (Fidy et al., 1998b). Because there are few thermally induced excitations and conformational transitions in the protein at this temperature, the illuminating laser light excites a certain subpopulation (in electronic transition energy) of the chromophores that experiences a frozen distribution of protein environments. Because of the selective excitation, the emission spectra are resolved; they contain sharp emission lines resulting from resonant vibronic excitations, superimposed on a background of broad bands also present due to emissions from nonresonant phonon-coupled excitations (Friedrich and Haarer, 1984; Fidy et al., 1998b). The spectra taken under the fine tuning of the excitation frequency allow for determining the vibrational levels of the electronically excited state, and the inhomogeneous distribution function (IDF) of the spectral origin (Kaposi and Vanderkooi, 1992). This function represents the true inhomogeneously broadened (0, 0) band shape function.

# RESULTS

## MgMP in DMSO

Two kinds of pressure experiments were performed with MgMP in DMSO. DMSO forms a solid glass around 20°C. In the first series, the pressure was applied at room temperature and the cell was cooled thereafter to cryogenic temperatures. The pressure was checked again, and the value measured at low temperature was used in the calculations. The pressure shift caused by the cooling was usually $<100$ MPa. These measurements, where the sample was *p*ressurized at *r*oom *t*emperature, will be referred to as PRT experiments. The results were compared to those obtained along the opposite temperature-pressure path: first cooled and *p*ressurized at *l*ow *t*emperature (PLT experiments). Both experiments were performed in the pressure range of 0–2.1 GPa.

### *Ambient pressure*

Fig. 1 shows the FLN spectra of MgMP/DMSO recorded at eight different excitation energies. The sharp (0, 0) electronic transition lines seen in the figure correspond with vibronic excitations of 592, 669, 730, and 896 $cm^{-1}$ within the excited electronic state. We collected series of similar emission spectra by scanning the excitation from 550 nm to 567 nm (18,180 $cm^{-1}$ and 17,640 $cm^{-1}$). The inhomogeneous distribution function was determined from the change of the intensity of the sharp emission lines while they shifted across the (0, 0) range by the tuning of the excitation frequency. Fig. 2 shows the IDF of (0, 0) lines at atmospheric pressure. Two bands can obviously be distinguished in this distribution, indicating two populations of the chromophores. The IDF was fitted accordingly with two Gaussians with maximum positions at 17,260 $\pm$ 15 $cm^{-1}$ and 17,020 $\pm$ 30 $cm^{-1}$, and half-width at half-height (HWHH) values of 100 $\pm$ 10 $cm^{-1}$ for both peaks.

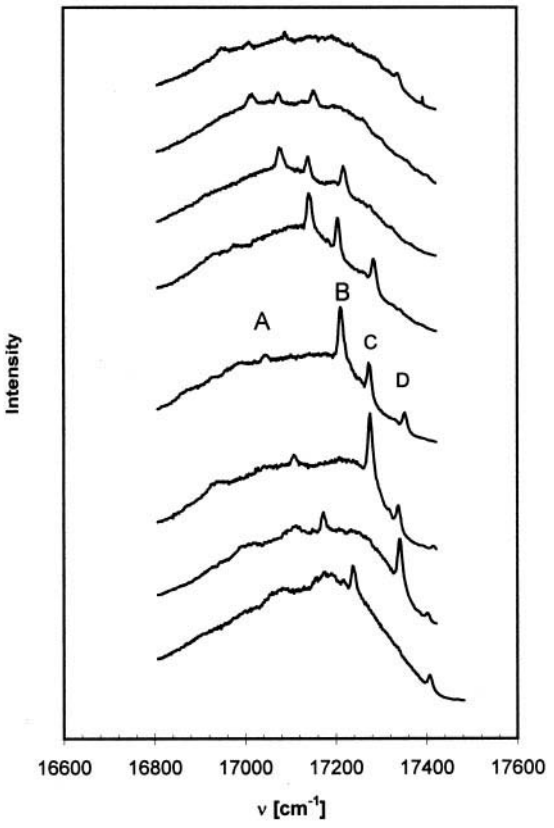



FIGURE 1 The FLN spectra of MgMP/DMSO (7 mM) measured at 10 K and ambient pressure. Excitations (from *bottom* to *top*): 551 nm (18,148 $cm^{-1}$) 553, 555, 557, 559, 561, 563, 565 nm (17,700 $cm^{-1}$). The lines *A*, *B*, *C*, and *D* belong to the 896, 730, 669, 592 $cm^{-1}$ vibrations of the excited state.

### *PRT experiments*

Fig. 3 *A* shows the IDFs of the MgMP/DMSO system in function of pressure. As the applied pressure was varied, the population of the two peaks was affected. The maximum position of the peaks also changed, it was slightly shifted to lower energies by increasing pressure. The relative area of the two peaks versus pressure is plotted on Fig. 3 *B*.

The FLN technique also allows determination of the vibrational energy levels of the excited state (Personov, 1983; Kaposi and Vanderkooi, 1992). In the 590–1200 $cm^{-1}$ energy range 12 vibrational levels were observed. The pressure dependence was determined for a few excited-state vibrations. The $\delta\nu/\delta p$ values were found to be 3 $cm^{-1}$/GPa, which are in the same order of magnitude as the usual shifts of the ground-state vibrational levels determined by Raman and infrared spectroscopy (Zakin and Herschbach, 1986; Heremans et al., 1996; Goossens et al., 1996).

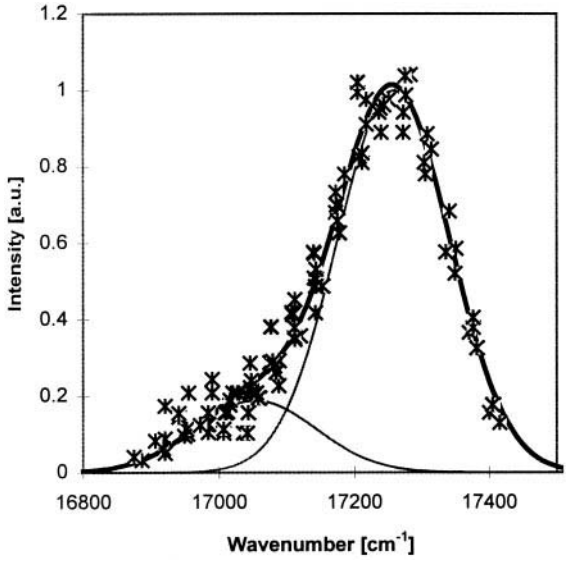
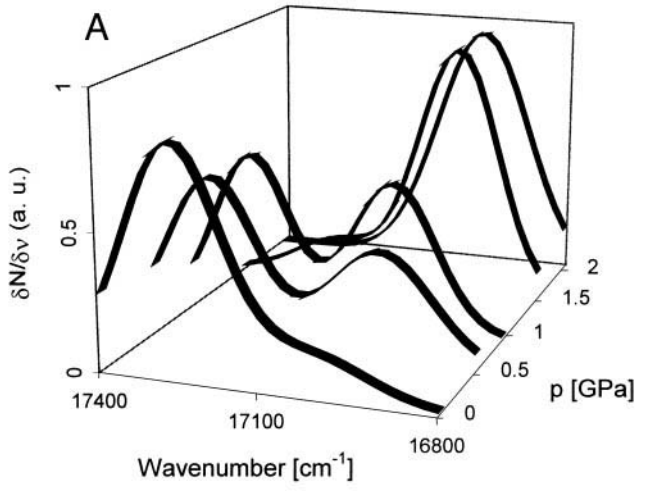



FIGURE 2 The inhomogeneous distribution function of MgMP in DMSO at ambient pressure, determined from measurements like the ones shown in Fig. 1.



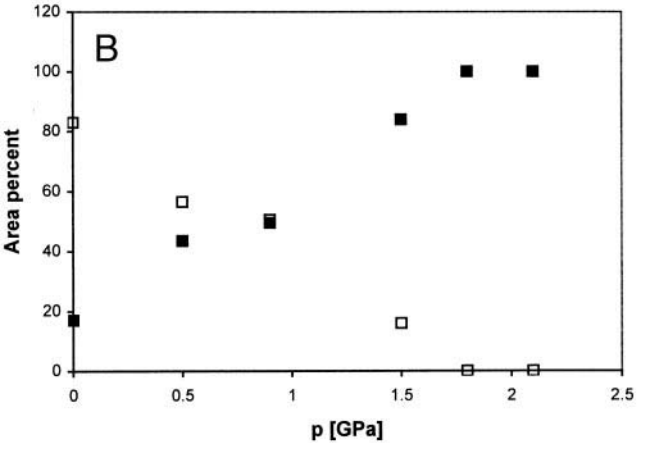




FIGURE 3 The effect of pressure when applied at room temperature on the inhomogeneous distribution function of the MgMP in DMSO matrix at 10 K. (*A*) The change of the IDF as function of the pressure; (*B*) integrated areas of the component peaks of the IDF versus pressure. Filled symbols belong to the peak at lower wavenumber, hollow ones to the peak at higher wavenumber. See experimental conditions, Fig. 1.

### *PLT experiments*

When the pressure was applied at low temperature, the IDF remained similar to the one measured at ambient pressure (≈0 GPa within the error of the pressure denaturation mentioned in the Materials and Methods section) (Fig. 2.). There was no change in the population of the two maxima under pressurization. The shift of the whole IDF was, however, observed, similarly to the PRT experiments. The shift of the main component versus pressure is plotted in Fig. 4. The pressure-induced shift of the smaller component was comparable with the shift of the major component, but the exact value could not be determined with sufficient accuracy because of the small intensity and overlap with the major band.

## MgMP-HRP

In the case of the protein samples, measurements at room temperature cannot be performed up to a pressure high enough to observe the pressure-shift effect, because HRP denatures around 1.1 GPa (Smeller et al., 1996). Thus, only the PLT protocol could be applied. The IDF of MgMP in HRP reveals mostly one component at ambient pressure in the protein matrix, as seen in Fig. 5. When applying high pressure, the inhomogeneous distribution function of the dominating population shifts to lower energies. Fig. 5 also shows the IDF of MgMP-HRP at 0, 0.6, 1.0, and 1.6 GPa. No intense new peaks emerged in the distribution function as the consequence of the pressurization. The shift of the distribution function is considerable, much more significant than that observed in the case of the DMSO matrix. The maximum position of the IDF versus pressure is plotted in Fig. 6. Besides the shift, a broadening of the distribution function was also observed. This effect becomes clearly visible above 0.6 GPa.

# DISCUSSION

## DMSO matrix

The IDF of purely electronic (0, 0) transitions of MgMP embedded in DMSO matrix has a bimodal distribution at zero applied pressure. Apart from the main conformation, which is populated by >80% of the molecules, a smaller band in the IDF indicates the existence of a conformation that is less favored at ambient pressure. This latter band is

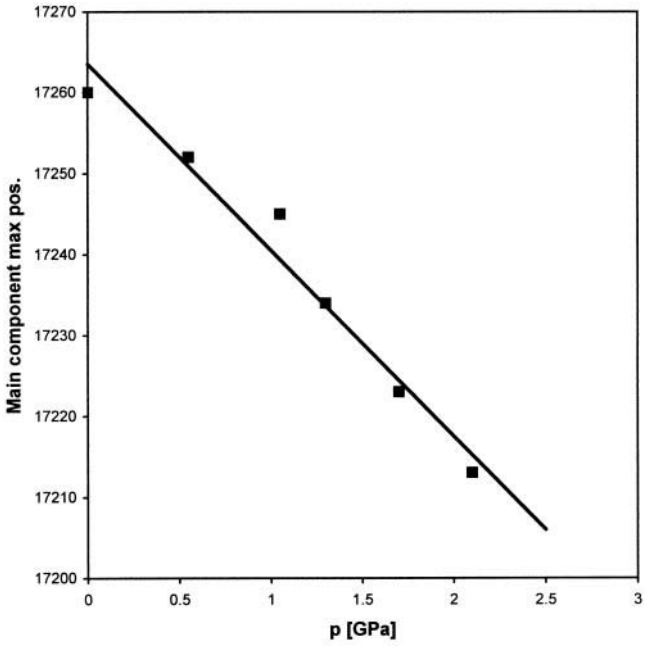



FIGURE 4 Maximum position of the main component of the IDF of MgMP in DMSO versus pressure, when the sample was pressurized at 10 K. MgMP concentration is 7 mM.

to the red compared to the main peak by ~250 $cm^{-1}$. The two bands appearing in the IDF indicate the presence of two distinct conformations of the chromophore-matrix complex in this system. The presence of more than one conformers was also found by Kaposi et al. (1993) in the case of a similar chromophore (Mg-protoporphyrin) in several organic solvent matrices. The splitting between the two components of the IDF (250 $cm^{-1}$) also corresponds to the value that can be estimated from the data in this paper for DMSO. It was reported by Platenkamp et al. that Mg-porphyrins are readily ligated to various components—even to organic impurities of the solvent (Platenkamp and Noort, 1982; Platenkamp, 1982). Ligation may change the energy of the electronic excited state, thus leading to a new (0, 0) band position.

The IDF changes under pressure, depending on whether the pressure was applied before (PRT protocol) or after (PLT protocol) the cooling of the sample to 10 K. In the case of the PRT protocol, the distribution of the chromophores in the two subpopulations also changes under pressure. The pressure-induced change in the population can be interpreted on the basis of the different volumes occupied by the distinct species. Suppose a Boltzmann distribution

$$\frac{A_h}{A_l} = \frac{N_h}{N_l} \propto e^{-(p\Delta V/RT)} \quad (1)$$

Where $A_h$ and $A_l$ are the areas of the higher and lower energy bands of the IDF; $N_h$ and $N_l$ are the number of molecules belonging to the corresponding subpopulations; $\Delta V$ is the volume difference between the two conformations ($\Delta V = V_h - V_l$); and other symbols have their usual meanings. To apply this formalism, the plot of $\ln(A_h/A_l)$ versus pressure was fitted with a straight line. From the slope $\Delta V/RT = 2.3 \pm 0.3$ $GPa^{-1}$ is obtained. The resulting $\Delta V$ is in the range of $0.19 \pm 0.03$ $cm^3$/mol ($T = 10$ K) to $5.6 \pm 0.8$ $cm^3$/mol ($T = 300$ K) depending on the temperature used in the calculation. The temperature, which can correctly be substituted into Eq. 1, is the lowest temperature, where the two conformational states of MgMP are in thermal equilibrium. Since the experiments performed along the PLT protocol did not show any repopulation of the states, this temperature should definitely be higher than 10 K. To estimate the lowest temperature, where the conformations can be interconverted by pressure, we performed experiments where the sample was cooled first to a certain temperature ($T_p$) where the pressurization took place. After pressurization to 2 GPa the sample was further cooled to 10 K to record the FLN spectra. Experiments at $T_p = 100$ K and 150 K showed no conversion, while the sample pressurized at $T_p = 200$ K was similarly converted as the sample pressurized at room temperature (data not shown). Taking into account these results, the volume difference between the conformers can be estimated as $3.3 \pm 0.7$ $cm^3$/mol (using $T = 175 \pm 25$ K). This corresponds to 5.5 $Å^3$, which is roughly the volume of six hydrogen atoms, or one-fifth of the volume of a water molecule (30 $Å^3$). The volume change is more than one order of magnitude smaller than the characteristic volume changes detected at the unfolding of proteins (50–100 $cm^3$/mol).

The electronic transition energy of the conformation with greater volume is higher. This is the favored conformation at ambient pressure. The position of the IDF bands characteristic for the two conformations can provide information about the strength of the interactions between the chromophore and its environment.

It can be generally supposed that the electronic transition frequency of the chromophores in a specific matrix is different from that in the absence of interaction (vacuum) (Laird and Skinner, 1989). The latter value is called vacuum frequency ($\nu_{vac}$). Balog et al. (1997) determined the vacuum frequency of MgMP in HRP by spectral hole-burning experiments: $\nu_{vac} = 17{,}560$ $cm^{-1}$. The environmental perturbations cause a shift (solvent shift) and it may also cause a broadening of the spectral lines. Both the broadening and the shifting effects can be characterized by the IDF. The two maxima of the IDF found in our experiments in the case of MgMP in DMSO reflect two conformations of the molecule with different solvent shifts or with different vacuum frequencies (or both). If we suppose identical vacuum frequencies (identical molecular conformations), the shift caused by the environment will be roughly two times larger for the conformation of lower volume. It seems reasonable that pressure would induce a transition to this conformation

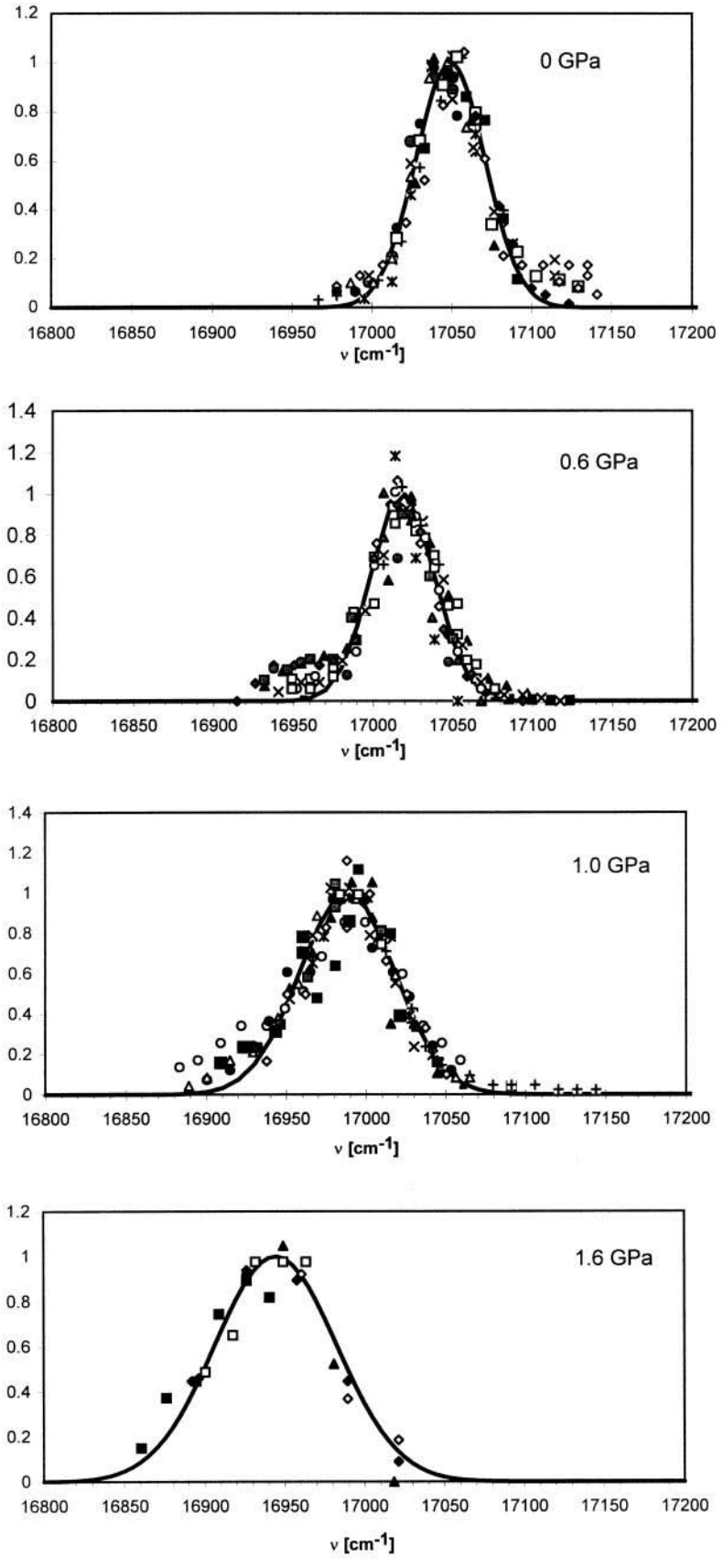



FIGURE 5 The IDF of MgMP-HRP 1.6 mM in 10 mM pH 7 phosphate buffer and 50% glycerol, at 0, 0.6, 1.0, and 1.6 GPa.

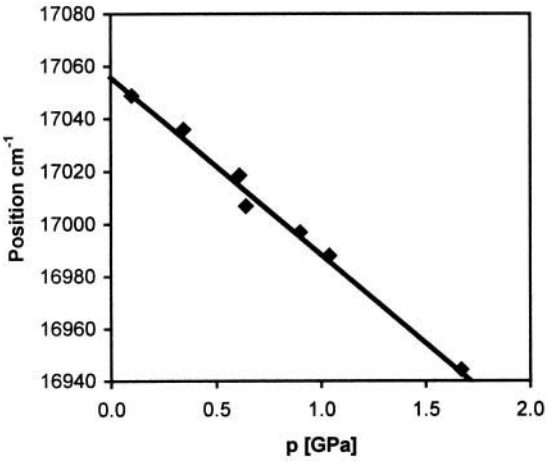



FIGURE 6 Maximum position of the IDF of MgMP-HRP as function of the pressure (see experimental conditions, Fig. 5.).

where the solvent interaction seems to be stronger, i.e., a better packing of the solvent molecules in the neighborhood of the chromophore is fulfilled. This model corresponds to the observation that the volume of the system is decreased by the pressure-induced transformation. The conformation, which allows better solvent packing, however, may not be entropically preferred, since at ambient pressure it is less populated.

The pressure-induced shift of the component peaks is very difficult to detect in the case of the DMSO matrix because the component peaks of the IDF are quite wide (HWHH is on the order of 100 $cm^{-1}$) and the shift is small. Weighted linear fit gives an estimate as $26 \pm 13$ $cm^{-1}$/GPa for the pressure-induced shift of the conformation with higher volume. For the other conformation the shift cannot be determined with reasonable precision.

### *Determination of the compressibility from the FLN experiments*

The pressure-induced shift of spectral lines was theoretically interpreted by Laird and Skinner (1989). According to this theory, the shift of a spectral line of an optical transition at a wavenumber $\nu_0$ to $\nu_p$ due to an applied pressure $p$ is proportional to the solvent shift ($\nu_0 - \nu_{vac}$) of the environment. ($\nu_{vac}$ is the so-called vacuum frequency, the wavenumber, where the chromophore would absorb light in the absence of environmental interactions, but with unchanged conformation.) In a linear approximation and supposing that the effect of the environment can be ascribed by the attractive part of the Lennard-Jones potential as $R^{-6}$, the model leads to the formula:

$$\nu_p - \nu_0 = 2(\nu_0 - \nu_{vac})\kappa p \tag{2}$$

Where $\kappa$ is the compressibility of the environment of the chromophore, supposing a homogeneous matrix. The model was widely tested by spectral hole-burning for a variety of matrices and chromophores and in many cases an agreement with the experimental data was found (Sesselmann et al., 1987a, b; Gradl et al., 1991; Pschierer et al., 1993). In his recent papers Renge (2000a, b) reported deviations from the theory in the case of certain chromophores embedded in a polymer matrix. The pressure effect was also studied in protein samples in a few cases (Zollfrank et al., 1992; Gafert et al. 1993; Köhler et al., 1996) including hole-burning studies on HRP (Gafert et al., 1994; Balog et al., 1997). In the low-pressure range, up to 2 MPa, the spectral shift data perfectly followed the relation predicted by the theory (Eq. 2) and the determined isothermal compressibility values were found to be independent of the chromophore (Balog et al., 2000) and sensitive to the conformation of the protein environments (Fidy et al., 1992, 1998b) The isothermal compressibility also of MgMP-HRP has been determined in the low-pressure range (Fidy et al., 1992; Köhler et al., 1998).

In the present experiment, not a single quasi-homogeneous spectral line, but their inhomogeneously broadened distribution, the IDF was determined. Thus we have to consider also the distribution of solvent shift values. Assuming a normal distribution $G(x)$, one can write:

$$\left.\frac{dN(\nu)}{d\nu}\right|_{\nu_0} = N_0 G(\nu_0) = \frac{N_0}{\sqrt{2\pi}\sigma} e^{-[(\nu_0-\nu_{00})^2/2\sigma^2]} \tag{3}$$

where $\nu_{00}$ is the center and $\sigma$ is the width of the IDF at zero pressure, and $N(\nu)$ is the number of the molecules with transition frequency greater than $\nu$. Since we know the function $f$ connecting $\nu_p$ and $\nu_0$:

$$\nu_p = f(\nu_0) = \nu_0 + 2(\nu_0 - \nu_{vac})\kappa p \tag{4}$$

the distribution function of $\nu_p$ can be calculated (Korn and Korn, 1968):

$$\left.\frac{dN(\nu)}{d\nu}\right|_{\nu_p} = G(f^{-1}(\nu_p))\left|\frac{df^{-1}(x)}{dx}\right| \tag{5}$$

giving:

$$\left.\frac{dN(\nu)}{d\nu_p}\right|_{\nu_p} = \frac{N_0}{\sqrt{2\pi}\sigma_p} e^{-[\nu_p-\nu_{0p}{}^2/2\sigma_p^2]} \tag{6}$$

where

$$\nu_{op} = \nu_{00} + 2\kappa p(\nu_{00} - \nu_{vac}) \tag{7}$$

and

$$\sigma_p = (1 + 2\kappa p)\sigma. \tag{8}$$

This calculation shows that the maximum of the IDF shifts under pressure the same way as the individual homogeneous lines do. This is in accordance with the finding of Laird and Skinner (1989) who also determined the shifting effect of pressure on the inhomogeneous line. A corresponding

broadening of the IDF should also be expected according to Eq. 8 if the same model is valid for both effects. Pressure-broadening was also studied in spectral hole-burning experiments and the results showed that in general, the broadening might be a more complex phenomenon than described by the above model (Pschierer et al., 1994).

### *Compressibility of DMSO*

The experiments performed according the PLT protocol resulted in the shift of the IDF component peaks. No repopulation between these peaks was observed; the ratio of the peaks remained unchanged within the experimental error in the whole pressure range (0–1.7 GPa). Both peaks seem to shift in the same way, but only the shift of the more populated one could be fitted with sufficient precision. The linear fit of the maximum position versus pressure yields $\partial\nu/\partial p = -23 \pm 2$ cm$^{-1}$/GPa as the slope of the straight line. From Eq. 8 one gets:

$$\kappa = \frac{1}{2}\frac{\partial \nu}{\partial p}(\nu_{00} - \nu_{\mathrm{vac}}) \tag{9}$$

where $\partial\nu/\partial p = \nu_{0p} - \nu_{00}/p$. Substituting the value given above for the slope, the compressibility value is $\kappa = 0.042 \pm 0.004$ GPa$^{-1}$. Compressibility values of DMSO are available only in the liquid state. Recent NMR measurements (Czeslik and Jonas, 1999) provided the parameters of the Tait equation in the temperature range of 30–70°C. The compressibility values that can be calculated from these parameters for the liquid phase are by roughly one order of magnitude higher than the values estimated by us for the glassy state. Taking into account the structural differences of the liquid and glass, this difference seems quite reasonable.

The slope $\partial\nu/\partial p$ determined from the PRT experiments—as shown above—is in agreement with the slope obtained from the PLT experiments, only the experimental error was larger. This suggests that the path followed on the *T-p* plane is not important for the determination of the compressibility of an amorphous solid. One can conclude that the effect of pressure in the elastic range is similar at room temperature and at cryogenic temperature. This observation underlines the significance of the data obtained at low temperatures. The fact, however, that the interconversion between the two peaks of the IDF was not observed at low temperature supports the idea that these peaks represent two conformationally different forms of the chromophore molecule, separated by a certain energy barrier.

Equation 8 predicts the broadening of the IDF maxima. This broadening was observed in both bands. The Gaussian, fitted to the main peak of the IDF, is broader by roughly one-fourth at 2.1 GPa compared to that at ambient pressure. The broadening rate was $11 \pm 1$ cm$^{-1}$/GPa. One can also calculate $\sigma_p/\sigma$ by substituting $\kappa$ from above into Eq. 8; it yields $\sigma_p/\sigma = 1.2$, which is in good agreement with the measured value within experimental error.

## Compressibility of MgMP-HRP

In contrast to the environment in DMSO, the IDF of MgMP in the protein HRP has only one major maximum. (The much smaller second maximum is neglected in the present studies, a detailed discussion about the origin of this band is given by Balog et al., 2000). This shape of the distribution function indicates that MgMP is in a well-defined environment when embedded in the heme pocket of HRP. The structurally well-defined environment is also reflected by the width of the distribution function. The IDF is considerably narrower in the case of the protein environment (HWHH $\approx$ 25 cm$^{-1}$) compared to DMSO (HWHH $\approx$ 100 cm$^{-1}$).

When pressurizing the MgMP-HRP sample at 10 K (PLT protocol) no new components were populated, the IDF remained of one component. In agreement with the above-explained theory of pressure effect, both a clear shift (Figs. 4 and 5) and broadening (Fig. 4) of the IDF were observed. The broadening rate for the protein was again $11 \pm 3$ cm$^{-1}$/GPa, supposing linearity in the whole pressure range. It was found, however, that the broadening became more effective toward higher pressures.

The pressure-induced shift of the maximum position could well be fitted by a straight line, and a slope of $67 \pm 3$ cm$^{-1}$/GPa was obtained. Using this value and the vacuum frequency ($\nu_{\mathrm{vac}} = 17{,}560$ cm$^{-1}$) determined by Balog et al. (1997) we obtained the compressibility $\kappa = 0.066$ GPa$^{-1}$. The related broadening rate is smaller than that obtained experimentally. This suggests that the model described above is much too simplified to also account for the phenomenon of pressure-broadening in proteins.

The value $\kappa = 0.066$ GPa$^{-1}$ is definitely smaller than the compressibility determined from spectral hole-burning experiments as 0.106 GPa$^{-1}$ (Table 1) for the same sample (Balog et al., 1997). Similar values were found earlier by the same technique on HRP with different substitutions and also on other hemoproteins (Zollfrank et al., 1991; Gafert et al., 1993) in the pressure range of 1–2 MPa. This higher value agrees very well with the overall isothermal compressibility estimated for deoxymyoglobin from normal mode analysis as 0.0937 GPa$^{-1}$ by Yamato et al. (1993), in a pressure range up to 100 MPa. In this later work, however, much lower isothermal compressibilities ($\sim$0.01 GPa$^{-1}$) were estimated for intrahelix compressibilities. Such lower values were also reported earlier for compressibilities in $\alpha$-helices, based on x-ray crystallography (Kundrot and Richards, 1987). The present measurements were carried out in a much broader pressure range, while the "fine tuning range" used in the spectral hole-burning experiments could not be observed at all. Our highest pressure value where the IDF was determined is 1.7 GPa, which is by three orders of

**TABLE 1 Isothermal compressibility of HRP determined by different methods**

| Method | $\kappa$ ($GPa^{-1}$) | Experimental Conditions | | Reference |
|---|---|---|---|---|
| | | $T$ (K) | $p$ (GPa) | |
| FLN | 0.066 | 10 | 0.1–1.7 | This work |
| Spectral hole burning | 0.106 | 1.5 | <0.0011 | Balog et al. (1997) |
| Ultrasound velocity | 0.067 | 298 | NA* | Gekko and Hasegawa (1986) |

*Typical pressure used in ultrasound experiments is <1 MPa.

magnitude higher than the pressures used in the cited spectral hole-burning experiments. Our interpretation of the results is that the compressibility at lower pressures is determined by the most compressible regions of the protein, while at high pressures these regions are already compressed, and therefore become more rigid. The rigid clusters may determine the compressibilities at higher pressures. Fig. 7 schematically shows this model. This interpretation is supported by results of recent molecular dynamics simulations on HRP in our laboratory (Schay et al., 2001) that showed internal cavities evolving around the heme site, and increased fluctuations of the electric potential around the active center of the enzyme. This pressure-tuned elasticity of the protein regions explains the difference between the results of the present measurements performed in the high-pressure range, 0.1–1.7 GPa, and the hole-burning measurements in the low-pressure range, 0.1–1.1 MPa. It is observed not only for proteins, but also for several organic polymers that the absolute value of the slope of the volume versus pressure curve decreases, which means that the compressibility decreases under pressure (Weber and Drickamer, 1983).

Ultrasonic velocity measurements at room temperature provided the adiabatic compressibility value of 0.0236 $GPa^{-1}$ (Gekko and Hasegawa, 1986). Taking into account the heat capacity ($c_p$) and the thermal expansion ($\alpha$), one can calculate the isothermal compressibility. Gekko and Hasegawa used $c_p$ and $\alpha$ values that were averages for several other proteins. Their isothermal compressibility value 0.067 $GPa^{-1}$ agrees very well with the value we obtained in the present work from FLN spectroscopy at cryogenic temperatures. The comparison of our results and those reported earlier based on various theoretical and experimental approaches also suggest that lowering the temperature does not significantly influence the isothermal compressibility in proteins and in organic solids.

## CONCLUSIONS

The high-resolution fluorescence line-narrowing (FLN) method combined with high pressure is capable of unraveling important information about proteins through the coupling of the monitoring porphyrin and the surrounding matrix. In the present study this effect was used to determine the isothermal compressibility from the pressure-induced shift of the inhomogeneous distribution function of Mg-mesoporphyrin in horseradish peroxidase and in DMSO. The compressibility of the protein was higher (0.066 $GPa^{-1}$) than that of DMSO (0.042). A pressure broadening was observed in both cases. The excited-state vibrational frequencies also showed a pressure shift similar to that found in the ground electronic state by resonance Raman and IR studies. The comparison of the compressibility of the protein obtained in the present study and in a range of much lower pressures shows evidence for inhomogeneities in the structure of proteins: the protein is more compressible at lower pressures. In the case of MgMP studied in DMSO matrix in its solid state, high pressure applied above ~175 K is able to change the conformation into another specific configuration predetermined by the matrix. This slight volume change, however, is able to significantly change the relative electrostatic field around the chromophore because the consequence is measurable by optical spectroscopy.

The data show that the compressibility does not depend significantly on the temperature either for the solid state amorphous matrix DMSO, or for the protein.

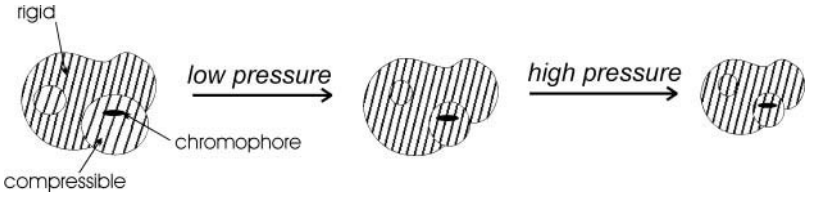



FIGURE 7 Schematic model of the pressurization of a protein consisting of regions of different compressibility. Low pressure predominantly squeezes the soft compressible domains, leading to higher compressibility, than that at high pressure, where the soft regions are almost completely compressed.

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