Spectral hole burning study of protoporphyrin IX substituted myoglobin

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ABSTRACT Protoporphyrin IX substituted myoglobin reveals excellent hole burning properties. We investigated the frequency shift of persistent spectral holes under isotropic pressure conditions in a range from 0 to 2.4 MPa. In this range, the protein behaves like an elastic solid. The shift of the holes under pressure shows a remarkable frequency dependence from which the compressibility of the protein can be determined. The compressibility, in turn, allows for an estimation of the equilibrium volume fluctuations. Within the frame of the model used to interpret the pressure data, it is possible to determine the absorption frequency of the isolated chromophore and the associated solvent shift in the protein environment.

INTRODUCTION

The technique of persistent hole burning (1) has been called the optical analogue of the Mössbauer-spectroscopy (2). There are several reasons which justify this analogy: first, both techniques are capable of working at the resolution limit given by the natural width of the respective transition. Second, there is an inherent symmetry between space and momentum in the Hamiltonian of the harmonic lattice, which leads to a very similar spectral pattern in both techniques, namely a sharp line superimposed on a background of phonons. From a practical point of view the respective resolving powers differ by some orders of magnitude: in the optical case $E/\Delta E$ is, for molecular solids, on the order of 10^8, whereas in the Mössbauer case it is on the order of 10^{12}. For singlet–singlet transitions in complex molecules a resolving power of 10^8 is already close to the natural lifetime limit. The extreme resolution power in both techniques is very attractive for investigating small internal or external perturbations on the probe transition considered. In the hole burning technique, in addition, one exploits the fact that the hole can be considered as persistent on proper time scales. Hence, it is possible to measure inhomogeneous line broadening effects on the frequency scale of the homogeneous width with high accuracy. Such inhomogeneous line broadening phenomena can be brought about by internal perturbations, such as structural changes in the neighborhood of the probe, which influence the probe transition and which lead to so-called spectral diffusion effects (3). Or, they can be brought about by external perturbations, e.g., magnetic and electric (4, 5) or strain fields (6, 7). Because the hole is so narrow, these fields can be quite small.

In this paper we focus on external strain fields which we induce by putting isotropic pressure onto the sample. The sample is a globular protein, namely myoglobin, in which the heme chromophore is substituted by protoporphyrin IX to enable hole burning. We stress that though we are not working at the resolution limit, the technique is so sensitive that pressure changes on the order of 0.1 MPa can be easily detected via a change of the parameters of the hole. At such low pressures, the protein responds fully elastic.

What kind of information do we get from a pressure tuning hole burning experiment?

If one restricts oneself to the shift of the hole under pressure, there are three quantities which can be determined. These are: the vacuum frequency of the chromophore, i.e., the frequency of the chromophore where it would absorb if deprived from all the surrounding, the solvent shift and the compressibility of the matrix. We will show below, that under certain conditions, the compressibility of the matrix can be related to the compressibility of the protein itself. Once the compressibility of a protein is known, its relative volume fluctuations can be determined. The size of the volume fluctuations is important with respect to the functioning of these kind of proteins. Myoglobin is, like hemoglobin, a transport protein for oxygen. The oxygen molecule is transported within the heme pocket. It can enter the pocket because the protein molecule performs once in a while large scale motions which open and close the pocket (8).

BASIC FEATURES OF PHOTOCHEMICAL HOLE BURNING IN PROTEINS

There is a series of chromoproteins, mainly chromoproteins of photosynthesis, which undergo persistent spec-

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tral hole burning reactions, so that narrow holes can be burnt into respective absorption bands. As far as native heme proteins are concerned, the situation is different. There is no narrow bandwidth photochemistry. Irradiation, for instance into the 579 nm (17,270 cm^{-1}) band of carbonmonoxymyoglobin, leads to an almost complete bleaching of the whole band (9). The most obvious reason for this is that the heme transition is largely homogeneously broadened due to fast intersystem crossing processes. These fast processes are promoted by the strong spin orbit coupling at the iron center. Pahapill and Rebane (10) determined the lifetime of the 582-nm transition in sperm whale oxymyoglobin to ~65 fs, which corresponds to a hole width of 160 cm^{-1}. This comprises the major part of the whole inhomogeneous line.

To overcome this obstacle, we substituted the heme chromophor by protoporphyrin IX (Fig. 1). Porphyrins are among the best known hole burning systems. The photoreaction in these systems is a proton transfer reaction. It has been assumed for long, that in this reaction, the inner protons of the ring are coherently rotated by 180° (11). Meanwhile, a lot of experimental evidence has been found which show that the situation is more complex. Several photoproduct states could be identified. For example, in mesoporphyrin IX substituted horseradish peroxidase, four such states were found (12, 13); in phthalocyanine doped *n*-octane, at least three could be identified (14). As to the nature of these states, there are several possibilities. Bersuker and Polinger (15) suggested that these states are formed through out of plane configurations of the protons involved. This way, four states can be constructed. However, it is also conceivable that the molecular plane is conserved as a symmetry element and that the protons reside in neighboring positions (16), in addition to the two diagonal configurations. In this case, even six configurations can be constructed. As of yet, there is no unambiguous evidence in favor for one of the two possibilities. However, it is for sure that more than two states are involved in the light induced switching of the two inner protons.

As to the transformation itself, it is known that it occurs during intersystem crossing into the lowest triplet state. From the spectroscopy of porphyrins (17) and phthalocyanins (18) in Shpol'skii crystals, it is known that the various phototransformed states differ in their absorption energies by some 50–100 cm^{-1}.

The S_1-lifetime of porphyrins is on the order of several nanoseconds. Hence, the natural width of these molecules is in the 10–100 MHz range. This means that at temperatures of 1 K or so, the photochemical holes burned into the inhomogeneously broadened absorption

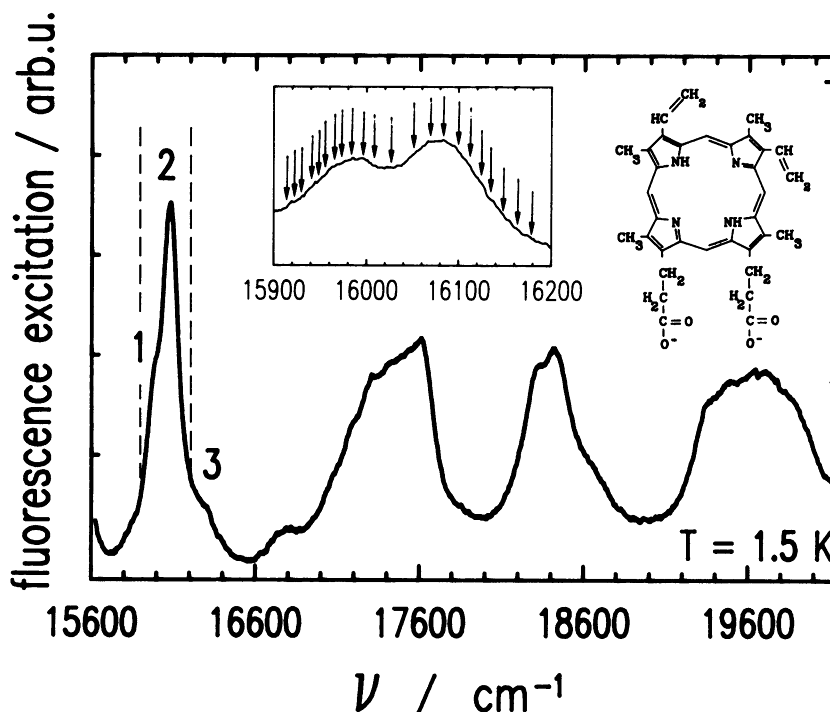

FIGURE 1 Fluorescence excitation spectrum of protoporphyrin IX substituted myoglobin. The insert shows an enlarged view of the part of the spectrum between the dashed lines. Hole burning positions are indicated by arrows. Solvent: Glycerol/water.

bands can be as narrow as that. However, there usually are additional broadening mechanisms (1) so that the fluorescence lifetime limit is reached only when special efforts are made. In our case, the holes are burned to a relative depth on the order of 30–50%. Hence, saturation broadening is dominant. This is an artificial broadening which can be reduced a lot when the irradiated energy is reduced, i.e., when the holes are less deeply burnt. Because we focused on pressure phenomena only in this study, the starting width is of no concern as long as the pressure induced changes are large compared to it. Hence, we burned rather deep holes to get a good signal to noise ratio. The width of the burned holes was on the order of 700 MHz when external pressure was absent.

PRESSURE EFFECTS IN PERSISTENT SPECTRAL HOLES

Application of pressure changes a hole in a three-fold way: there is a frequency shift, there is a broadening, and there is a change of its shape. All these changes can be understood on the basis of recently developed theories on inhomogeneous line broadening phenomena and related pressure effects (19, 20). In this paper we focus on the pressure induced line shift from which the above mentioned three quantities, namely the vacuum frequency of the chromophore, the solvent shift and the compressibility, can be determined.

Proteins are very often interpreted on the basis of glass models (21–25). In the following, we adopt this view and treat the environment of the chromophore as a glassy medium. We do not, at this level of treatment, distinguish between protein and host glass. Implications of this treatment are discussed later.

Suppose a solvent atom at distance R from the chromophore leads to a change of the free chromophore absorption by an amount of $v(R)$. When pressure is applied, $v(R)$ changes by $\Delta v(R)$. Because the applied pressure is so small, a first order expansion is sufficient. We get

$$\Delta v(R) = \frac{\delta v(R)}{\delta R}\frac{\delta R}{\delta p}\Delta p. \quad (1)$$

We presume that on sufficiently large scales the glassy medium can be considered as homogeneous and isotropic. Then, $\delta R/\delta p$ can be written as (19)

$$\frac{\delta R}{\delta p} = -\frac{R}{3}\kappa, \quad (2)$$

with κ being the compressibility. To arrive at quantitative results, one has to put in an interaction potential for $v(R)$. It has been shown by Laird and Skinner (19) that a modified Lennard-Jones potential is necessary to account for the pressure induced broadening of the holes. However, as far as the line shift is concerned, it suffices to consider the attractive part of the Lennard-Jones potential, i.e., the dispersion forces, only. The dispersion forces fall off as R^{-6}, hence, they have a much longer range than the repulsive part. Many more atoms of the lattice are involved in this type of interaction. On the other hand, the range of the repulsive interaction is so short that only the nearest neighbor shell of atoms is involved. The influence of the atoms in this shell on the line shift of the probe is partially compensated; hence, we neglect this shell. The interaction with the rest is governed by the dispersion interaction. Then, if $v(R) \propto R^{-6}$, we get from Eqs. 1 and 2,

$$\Delta v(R) = 2\kappa v(R)\Delta p. \quad (3)$$

Eq. 3 holds for a single solvent molecule. Our experiment is frequency selective. The hole burning process selects an ensemble of chromophores which absorb all at the same frequency v. Hence, all chromophores in this ensemble have the same solvent shift v_s, which is defined as

$$v_s \equiv v - v_{vac}, \quad (4)$$

with v_{vac} being the vacuum absorption frequency of the chromophore.

Note that in a frequency selective experiment, the solvent shift is a parameter of the experiment which depends on the excitation frequency within the inhomogeneous band.

To describe our experiment we have to average Eq. 3 over all solvent environments of the probe which generate exactly the same solvent shift. Then Eq. 3 becomes

$$\langle \Delta v \rangle = 2\kappa \langle v(R) \rangle_c \Delta p. \quad (5)$$

$\langle v(R) \rangle_c$ is the perturbation averaged over these environments. It is exactly this quantity which generates the solvent shift for the frequency selected subensemble considered

$$v_s \equiv \langle v(R) \rangle_c. \quad (6)$$

Using Eqs. 4–6 we have

$$\langle \Delta v \rangle = 2\kappa (v - v_{vac})\Delta p. \quad (7)$$

$\langle \Delta v \rangle$ is the measured shift of the hole burned at frequency v when pressure Δp is applied. The burn frequency v can be tuned over the inhomogeneous band. We learn from Eq. 7 that there is a frequency where the

pressure induced shift vanishes. This frequency is the vacuum absorption frequency, i.e., the frequency where the chromophore would absorb, if deprived from its environment. In some glassy systems, this frequency lies within the inhomogeneous band and, hence, can be measured directly (26). However, in most cases it has to be determined by extrapolating the burn frequencies (27–29). Once v_{vac} is known, the average solvent shift $\langle v_s \rangle$ (i.e., the solvent shift averaged over the whole inhomogeneous distribution or, what is equivalent, averaged over all possible solvent environments) can be determined:

$$\langle v_s \rangle = v_0 - v_{vac}. \tag{8}$$

v_0 is the maximum of the inhomogeneous band under consideration.

Furthermore, if the frequency shift per unit pressure $s \equiv \langle \Delta v \rangle / \Delta p$ is measured as a function of burn frequency v, one gets a straight line, whose slope is twice the compressibility. Hence, the compressibility can be measured by solely using optical techniques.

In this simple analysis of pressure tuning of optical holes, there are two severe approximations involved. (*a*) The matrix is assumed to be homogeneous and isotropic with respect to its elastic properties; and (*b*) there is no correlation among the solvent molecules. This means that each solvent molecule acts independently on the chromophore.

EXPERIMENTAL

The heme group of horse myoglobin was extracted with the help of butanon as described in (30). Also, the reconstruction followed but instead of heme, protoporphyrin IX was built in (30). The final sample was obtained by mixing 1 ml of the protein water solution (3.56 mg reconstituted myoglobin/1 ml) with 3 ml glycerol.

The holes were burned to a relative depth on the order of 30–50%. As mentioned above, holes of such a depth are severely saturation broadened. The burning laser was a ring dye laser, pumped by a 6 watt argon ion laser. The bandwidth of the dye laser was on the order of 2 MHz, its power level on the order of 200 mW. The laser frequency was measured with a wavemeter, based on a scanning Michelson interferometer with an accuracy of 1 cm^{-1}. The holes were measured by detecting the broadband fluorescence emission while the laser was scanned over the range of the hole. The scan range of the laser was confined to 30 GHz. In the scanning mode, the power level of the laser was reduced by a factor of 400 as compared to the burning mode.

The sample was sealed off in a small plastic bag. This bag assured that the sample did not get stuck to the windows of the pressure chamber during freezing. This way, we could ensure that the applied pressure was isotropic, indeed. The plastic bag was formed from two sheets of thin film which were welded together. Pressure was transmitted via He-Gas and could be measured with an accuracy of 10 hPa. The whole pressure cell was immersed in liquid He and kept at a temperature of 1.5 K. At 1.5 K, the maximum pressure used was 2.4 MPa. Liquid He solidifies at a pressure of 2.5 MPa at this temperature. To check the elastic response of the sample and a possible influence of pressure during freezing, the sample was exposed up to 8 MPa at 4.2 K.

We performed several different experiments: for the pressure shift experiments, the sample was cooled to 1.5 K. Then a hole was burned and exposed to pressure. For each pressure value, the hole was subject to a fit procedure with a Voigtian lineshape, from which the corresponding parameters were determined. This procedure was repeated for as many as 22 frequencies within the longest wavelength absorption band.

Because the above model on pressure phenomena in spectral holes is confined to elastic deformations, we checked in a separate experiment that only elastic forces are probed in the pressure range considered. In principle, nonergodic systems, like proteins and glasses, can undergo irreversible relaxation, e.g., conformational changes. As a matter of fact, such nonergodic relaxation behavior has been observed in pressure release experiments (31). However, in this case, the applied pressure was two orders of magnitude higher than in our case. To be sure that irreversible changes of conformations do not occur, we froze the protein solution under a pressure of 8 MPa. The pathways in the pT plane are shown in the insert of Fig. 2. Then, at 4.2 K, a hole was burned (3). After burning, pressure was released. The hole broadens so strongly that it disappears (4). Subsequently, a pressure of 8 MPa was restored again. As can be seen, the hole fully recovered (5). There are no traces of an irreversible change. Hence, we are sure that up to a pressure of 8 MPa the system behaves like an elastic solid.

RESULTS

Fig. 1 shows the overall spectrum of protoporphyrin IX substituted myoglobin. The frequencies, where hole burning was performed, are indicated by arrows. The low frequency band, on which we focused our experimental work, shows clearly a substructure. At least three different transitions marked with 1, 2 and 3 can be identified.

Fig. 2 shows that the application of pressure does not

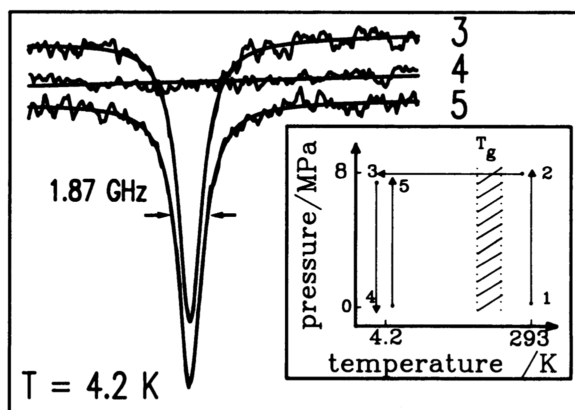

FIGURE 2 Cross-check of protein reversibility under applied pressure. The "pathway" along which the experiment is performed is shown in the insert: the sample is pressurized at ambient temperature up to 8 MPa (1). The pressurized sample is frozen to 4.2 K (2). A hole is burnt (3). Pressure is released (4). The hole completely vanishes. The sample is again pressurized (5). The hole fully recovers.

lead to irreversible conformational changes, as has been stressed above.

Fig. 3 demonstrates how a burned-in hole responds to the application of isotropic pressure. It shifts and broadens,

Fig. 4 shows two noteworthy results: first, the shift of the hole is perfectly linear with pressure. Second, there is a remarkable color effect as the burn frequency is varied across the inhomogeneous line. The shift per unit pressure (i.e., the slope of the straight lines in Fig. 4) is much stronger in the blue edge of the band than in red. We stress that for $\pi\pi^*$ excitations a blueshift is rather unusual.

Fig. 5 shows how the pressure induced line shift depends on burn frequency. Clearly, there are two well-defined frequency ranges, where the shift per unit pressure depends in a linear fashion on the burn

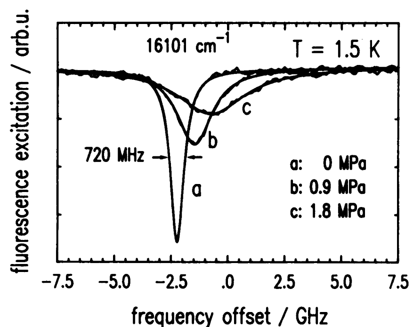

FIGURE 3 Behavior of a spectral hole under pressure.

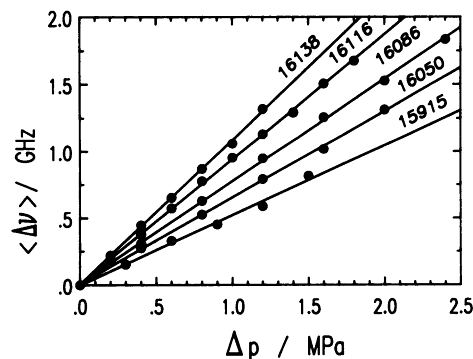

FIGURE 4 Pressure shift of spectral holes at various burn frequencies.

frequency. The slopes of the two lines are identical within the error margins of the experiment. Deviation from the linearity occurs in the region where bands 1 and 2 strongly overlap. Different symbols in the plot mean that the experiment was repeated three times. They perfectly fit together, which demonstrates the high degree of reproducibility of this type of experiment.

DISCUSSION

We start the discussion by stressing that the protein-glass system behaves perfectly as an elastic solid within the pressure ranges of the experiment (Fig. 2). This is, of course, a prerequisite to interpret the data according to the model outlined above.

Proteins are characterized by a huge manifold of conformational substates (22, 32). This means that in an

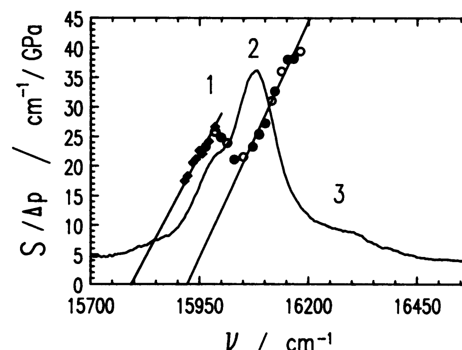

FIGURE 5 Frequency shift per unit pressure as a function of burn frequency. The straight lines represent least square fits to the data in the pure band regions. Their extrapolations to zero determine the vacuum frequencies $\nu_{vac,1}$ and $\nu_{vac,2}$ of the dye species. Their slope is twice the compressibility. The experiment was repeated three times. The corresponding data are represented with different symbols.

ensemble each molecule has a slightly different structure. The structural distribution reveals itself in the Debye-Waller factor of the x-ray structure analysis (33, 34). At temperatures as low as 1 K, each protein can be considered as being frozen in a particular substate. It is the structural distribution which brings about the similarities of proteins and glasses, which is, for instance, reflected in the inhomogeneous line broadening features observed. We may interpret the average in Eq. 6 as an average over a subensemble of these conformational substates, which leads to a specific solvent shift as selected by the hole burning frequency.

We found that the qualitative predictions of the model as outlined above are quite well reproduced by the experiments on proteins: The shift varies linearly with pressure (Eq. 5) and the corresponding slope factor $\langle \Delta v \rangle / \Delta p$ (the shift per unit pressure) varies linearly with burn frequency (Eq. 7). The slope factor 2κ of $\langle \Delta v \rangle / \Delta p$ as a function of burn frequency is the same for the two frequency ranges (Fig. 5), hence, seems to be a characteristic parameter for the material investigated. Though the model seems to work well on a qualitative level, we have to ask what the numbers we get out, namely v_{vac} and κ, really mean.

Vacuum frequency of protoporphyrin IX

The basic physics behind the simple model is reflected in Eq. 5, which states that the pressure shift of the hole is proportional to the solvent shift. Those probes which interact strongly with the environment experience a strong pressure shift, those which interact weakly experience a weak pressure shift. Because the solvent shift can be varied across the inhomogeneous band, an extrapolation of the data to a frequency with zero solvent shift is possible. This frequency is the vacuum absorption frequency ν_{vac} according to the definition given in Eq. 8. Though the proportionality between pressure shift and solvent shift seems plausible, it should be noted that, strictly speaking, it only holds for R^{-n}-type interaction potentials. Nevertheless, we stress that there are systems where the vacuum frequency can be measured directly (26) and not just by extrapolation. Hence, there is indeed a well-defined frequency where burned-in holes do not undergo any pressure shift at all, as suggested by the above model. As one scans the burn frequency across this range, the pressure shift changes sign. We consider this a strong indication that the model is quantitatively correct. Summarizing, we can say that there are many experiments on doped glasses, which support the view that a solid-state spectroscopy technique, namely pressure tuning of spectral holes, can provide information on parameters of the isolated probe species.

From Fig. 4 we see that upon application of pressure the hole shifts to the blue. Hence, according to Eq. 7, the vacuum frequency of the protoporphyrin chromophore is smaller than the burn frequencies, which means that the solvent shift is positive, i.e., the chromophore experiences a blue shift through the protein environment. A blue shift is, for $\pi\pi^*$-excitation, rather unusual. It is usually observed for $n\pi^*$-transitions. In general, it means that the molecule lattice interaction weakens upon excitation. As far as porphyrins are concerned, the admixture of $n\pi^*$ configurations into the lowest S_1-state has indeed been considered (35). However, it should be noted in this context that mesoporphyrin IX in horseradish peroxidase shows a redshift (28). Both molecules are identical except for the fact that the vinyl groups in protoporphyrin are substituted by ethyl groups in mesoporphyrin. With Eq. 7 we can determine the vacuum frequency of protoporphyrin from Fig. 5 just by extrapolating the measured frequency shift per unit pressure to the frequency where this quantity vanishes. Because we have two ranges where s varies in a linear fashion with v, we get two vacuum frequencies. They are 15,794 cm^{-1} and 15,922 cm^{-1} (± 20 cm^{-1}).

There are some noteworthy facts: the 16,000 cm^{-1} band has a clearly discernable substructure (bands 1, 2 and 3 in Fig. 1). We attribute this bands to different tautomeric forms of the porphyrin probe. As a matter of fact, there may be more than two tautomers because the two inner protons can attain a series of arrangements, as has been shown in the case of mesoporphyrin in horseradish peroxidase (12). One may argue that the bands could reflect a set of conformational substates of the apo-protein, as is, for example, observed in the infrared spectrum of carboxymyoglobin (32). If this were the case, we would observe two data sets with different slopes converging to a common vacuum frequency somewhere on the red side of the band. This is definitely not the case. Therefore the two vacuum frequencies belong to two protoporphyrin species, where the arrangement of the inner ring protons differ from each other. A third form of the probe should be responsible for band 3, which is too weak for pressure dependent investigation. We can also rule out the possibility that the substructure originates from a vibronic progression. The hole width is nearly the same for all burn positions and, hence, they do not reflect any vibrational relaxation.

These results demonstrate that pressure tuning hole burning spectroscopy is capable of measuring vacuum frequencies of chromophore molecules under as-is-conditions, i.e., under the conditions where the chromophores absorb in the actual state which it attains in the solvent, when deprived from all of its environment.

Although, for some systems the vacuum frequency can be measured when the probe can be seeded into a supersonic jet (36, 37), for many systems it is impossible to gain information on υ_{vac} with the jet technique. This situation prevails, for example, if molecules undergo protonation and deprotonation reaction or structural changes, when embedded into a solvent (27). In this case, pressure tuning spectroscopy is the only way to gain information on υ_{vac}.

Compressibility factor

The slope factor determined from a plot of the shift of the hole per unit pressure as a function of burn frequency, is twice the compressibility factor κ (Eq. 7). Again, we stress that this holds in an exact way only if the dominating probe-solvent interaction falls off as R^{-6}. As a matter of fact, a series of experiments on a variety of probe-solvent systems shows that an R^{-6} type interaction potential describes the systems in a quantitative fashion sufficiently well. This is even true for polymers (6, 7), which are, like proteins, subject to some restrictions as compared to organic glasses, through the covalent bonds along the backbone.

Then, from the slopes in Fig. 5, we determine κ to be on the order of 0.07 GPa^{-1}. This value for κ falls well into the range of low temperature compressibilities of organic glasses and polymers (6, 7, 26, 27). Table 1 gives an overview of compressibilities for a series of materials. Some of the polymer compressibilities were also measured by mechanical methods and fit well into the scenery. Also, the value of the only protein molecule, where pressure tuning experiments have been performed so far, namely mesoporphyrin IX substituted horseradish peroxidase, is close (28). An interesting observation is that, whereas the values for organic liquids vary markedly from ambient temperature to 1.5 K, the compressibility of proteins seems to be rather insensitive to temperature.

TABLE 1 **Compressibilities**

System	Compressibility/GPa^{-1}		Reference
	$T \approx 1.5$ K	$T \approx 293$ K	
Ethanol/methanol	0.18 (±0.01)	—	(27)
Ethanol	—	1.12	(41)
Methanol	—	1.22	(41)
Glycerol	0.10	0.22	(26, 42)
Ethylene glycol/water	0.12	—	(26)
Ethylene glycol	—	0.37	(41)
Water	—	0.46	(41)
Polystyrene	0.15	0.33	(6, 43)
Polymethylmetacrylat	0.15	0.24	(43)
Horse radish peroxidase	0.10	—	(28)
Substituted myoglobin	0.07	—	this work
Various proteins	—	0.06–0.12	(38)

The main question in this context is, however, whether we really probe the protein or whether the results of the experiments reflect just properties of the host glass. To answer this question, a specific feature of the optical technique comes in: in the optical technique, changes in the lattice upon compression are detected through the spectral properties of the dye probe. The dye probe, however, only feels the changes of those molecules which are within the range of the interaction forces. As to the dispersion forces, their interaction integrated over space falls off as R^{-3}. Assuming a nearest neighbor distance on the order of 3 Å or so, a shell of atoms at a distance of 3 to 4 equilibrium distances yields an almost negligible contribution. This means that the optical technique measures a local compressibility, i.e., the compressibility of a sphere with a diameter of ~15 Å around the dye. From x-ray data, it is well known that protoporphyrin IX resides within an apoprotein pocket with the two propionate groups extending into the surrounding solvent. Nevertheless, it seems reasonable to assume that the porphyrin ring mainly feels the pressure induced changes in the apoprotein and not those in the solvent. At least, it seems safe to assume that the contribution of the solvent is strongly reduced as compared to that of the apoprotein. We stress that the value obtained from our experiment is not necessarily equivalent to the compressibility of a protein crystal, because there, the intermolecular packing and interaction may play an appreciable role. However, we point out that the compressibilities of the two protein molecules which we have measured so far fall exactly in the range 0.06–0.12 GPa^{-1}, as estimated from sound velocity measurements on a series of protein solutions (38, 39) (see Table 1).

The elastic response to pressure and the similarity to glasses concerning the absolute values of the compressibility, shows that globular proteins are rather densely packed. There is no space for irreversible deformation within the pressure range of our experiment.

The compressibility provides information on the packing density and on the molecular interactions. However, what is more interesting for protein molecules, like myoglobin, is the fact that it determines the magnitude of the volume fluctuations. For transport proteins sufficiently large volume fluctuations are a prerequisite for a proper functioning (8). Once in a while, the protein opens pathways wide enough so that a substrate molecule like O_2 or CO can penetrate and can be bound to the heme iron.

The relative volume fluctuations of a system are determined by the compressibility, the temperature and

the volume V itself (see, for instance, 40):

$$\frac{\Delta V}{V} = \sqrt{\frac{kT\kappa}{V}}. \tag{9}$$

The volume of myoglobin is on the order of 10^4 Å^3. Hence, with the measured compressibility of 0.07 GPa^{-1} one calculates from Eq. 8 relative volume fluctuations at ambient temperatures on the order of 0.5%. This is the same order of magnitude which we found for mesoporphyrin substituted horseradish peroxidase (28). The lower κ-value in myoglobin is compensated by its smaller volume, so that the magnitude of the relative fluctuations is the same for both molecules. We stress that these are lower estimates because κ could somewhat increase with temperature. However, average volume fluctuations on the order of 0.5% provide enough space to open channels for small molecules like O_2 or CO to penetrate the myoglobin pocket.

SUMMARY

We performed pressure tuning experiments on photochemical holes burnt into protoporphyrin IX substituted myoglobin. We used pressure differences up to 8 MPa and found that within this pressure range the protein responds like an elastic solid. There is no influence of the pathway on which a certain point within the p–T plane is reached. Provided the interaction of the chromophore with its environment is homogeneous, isotropic and of the R^{-n}-type, it can be shown that the shift of the hole per unit pressure depends in a linear fashion on the burn frequency. The slope of the corresponding straight line is given by the compressibility of the system. We argued that this compressibility is the compressibility of the protein investigated. Its absolute magnitude fits into what is known about proteins in this context. In addition, the experiments allowed for a determination of the vacuum absorption frequency of the dye probe and the absolute magnitude of the solvent shift. In the case considered, this latter quantity is positive.

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