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Spectral hole burning and selection of conformational substates in chromoproteins

(horseradish peroxidase/compressibility/disorder phenomena)

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ABSTRACT **We investigated spectral holes burnt at 1.5 K into the origins of several tautomeric forms of mesoporphyrin IX-substituted horseradish peroxidase at pH 8 under pressures up to 2 MPa. From the pressure-induced lineshift the compressibility of the apoprotein could be determined. We found that the compressibility changed significantly when measured at different tautomer origins. It was concluded that there must be a correlation between the tautomer configurations of the chromophore and the actual structures of the apoprotein. As a consequence, specific conformational substates of the protein can be selected by optical selection of the associated tautomers.**

The solid-state physics of proteins is an intriguing field. Unlike crystals, proteins are finite systems, yet they have a smooth density of vibrational states which is Debye-like at sufficiently small energies (1). Like crystals, proteins are highly ordered (2). Yet disorder plays a very important role, too (3, 4). Disorder manifests itself in inhomogeneously broadened spectral lines, in nonexponential kinetics, in non-Arrhenius-type activated processes, in a glass-like specific heat, in dielectric damping, etc. (5–8). Even from x-ray scattering experiments, it became obvious that, at a sufficiently high level of resolution, the structure of a protein is not so well defined (2, 3). There seems to be agreement that a certain extent of structural disorder is a prerequisite for the proper functioning of a protein.

A possible model for describing the relation between order and disorder in proteins is based on the concept of conformational substates (4, 7, 9, 10): The basic idea of this model is that a protein can exist in a huge set of substates. Most of these substates are assumed to be in fast equilibrium because their separating barriers are sufficiently small compared with kT. Some of them, however, are nonequilibrium states. These special substates may be functionally important. It is structural disorder which gives enough freedom to the protein to reorganize its structure for specific requirements.

Here we address the problem of how this reorganization takes place and how this is related to the prosthetic group. Can a slight structural change of the prosthetic group, as induced by a weak chemical interaction, be a signal for the protein structure to be rearranged, for example, to help the binding of special molecules? That is, we address the problem of correlation between the configurations of the prosthetic group and the conformational substates of the protein.

The experimental technique we employ is persistent spectral hole burning (11–15). Hole burning is a special type of saturation spectroscopy. Its specific feature, as compared with similar techniques in NMR and ESR spectroscopy, is the persistence of the hole. This persistence is the reason why the technique can be used to study the ground state by optical means. At liquid-helium temperatures, the width of a spectral hole is close to the natural width of the optical transition involved. For many chromophores, the fluorescence lifetime is in the nanosecond regime. This means that the natural width of the transition is in the megahertz-to-gigahertz regime. Hence, compared with the inhomogeneous width of the absorption band, which may be on the order of several hundred wavenumbers in glasses, a hole may be orders of magnitudes narrower. It is this sharpness in addition to the persistence what makes the technique unique. Extremely weak perturbations imposed on the system can be investigated by observing the induced changes in the central frequency, the width, and the shape of the burnt-in hole. Weak perturbations can be external fields or internal structural changes (16–18). In our case, it is the external pressure that is varied.

We have chosen the free base mesoporphyrin (MP) as a chromophore for several reasons: (*i*) Replacement of heme by MP does not abolish the substrate binding activity of the enzyme horseradish peroxidase (HRP); i.e., MP is a model for a native prosthetic group. (*ii*) A well-known (11, 19, 20) photochemical reaction, the light-induced transfer of the two inner pyrrole hydrogens, makes MP an ideal chromophore for photochemical hole burning. (*iii*) The detection of the spectral holes can be done in the fluorescence excitation mode (20, 21), which provides a technical advantage. (*iv*) HRP is a widely studied enzyme with a well-protected central heme pocket. The inhomogeneous broadening of the phototautomers of MP-HRP is about 60 cm^{-1}. This is less than their associated energy differences. As a consequence, the various chromophore configurations can be separately investigated.

The model used to evaluate the hole-burning results has two parameters, the vacuum absorption frequency of the chromophore, and the isothermal compressibility of the protein (22). The first parameter characterizes the configuration of MP, and the other is a global parameter of the protein dependent on its structure. The results will show that MP-HRP has different compressibility values for different tautomeric forms. This surprising result fits beautifully into basic physical models of proteins and shows that there is a correlation between tautomeric configurations and protein conformations.

Spectral Holes and Pressure Phenomena

We will treat the protein as a homogeneous, isotropic medium which surrounds the chromophore. Note that the pressure phenomena are measured through the spectral properties of the chromophore (22–24). Since the chromophore can feel the compression of the environment only within a sphere roughly determined by the chromophore-lattice interaction, the properties measured this way are local properties. If the length scale of this interaction does not exceed the dimensions of the protein, the influence of the solvent can be neglected, and it is

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Abbreviations: HRP, horseradish peroxidase; MP, mesoporphyrin IX.

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the properties of the protein which are reflected in the specific spectral features of the hole under pressure.

A fully microscopic theory of pressure effects on spectral holes was recently developed by Laird and Skinner (25). Their model is based on a series of approximations: (*i*) the N-particle density function can be factored into a product of pair correlation functions involving the solute–solvent interaction only; (*ii*) the perturbation of the transition frequency through the solute–solvent interaction is pairwise additive; and (*iii*) the density of the solvent is high enough so that the time correlation function (and hence the lineshape function) can be approximated by a Gaussian curve. Under these assumptions, the pressure-induced shift of the hole is fully determined through the density ρ_0, the compressibility of the solvent κ, the solute–solvent pair correlation $g(R)$, and the perturbation $\nu(R)$ which a solvent molecule at distance R imposes on the absorption frequency of the probe molecule.

Suppose a hole is burnt into the sample under isotropic pressure conditions. Then the pressure is changed by an amount Δp.

As a consequence, $\nu(R)$ experiences a change,

$$\Delta\nu(R) = \frac{\delta\nu}{\delta R}\cdot\frac{\delta R}{\delta p}\Delta p \equiv \alpha(R)\Delta p. \qquad [1]$$

Since we assumed the sample to be isotropic and homogeneous, $\alpha(R)$ can be simplified to

$$\alpha(R) = \frac{-R}{3}\kappa\frac{\delta\nu(R)}{\delta R}. \qquad [2]$$

Within the frame of the above approximations, the pressure shift S and the pressure broadening σ of the hole are given by

$$S = \left[N\langle\alpha(\mathrm{R})\rangle + \frac{\langle\alpha(R)\nu(R)\rangle}{\langle\nu^2(\mathrm{R})\rangle}(\nu_b - \nu_0)\right]\Delta p \qquad [3]$$

and

$$\sigma = [N\langle\alpha^2(R)\rangle(1-\rho^2)]^{1/2}|\Delta p|. \qquad [4]$$

ρ is the degree of correlation between $\alpha(R)$ and $\nu(R)$:

$$\rho^2 = \frac{\langle\alpha(R)\nu(R)\rangle^2}{\langle\alpha^2(R)\rangle\langle\nu^2(R)\rangle}, \qquad 0 \le \rho^2 \le 1. \qquad [5]$$

ρ is a measure of the similarity between the pressure-induced changes of $\nu(R)$ with $\nu(R)$ itself.

The angle brackets indicate ensemble averages; e.g.,

$$\langle\alpha(R)\rangle = \frac{1}{V}\int d\mathbf{R}\, g(R)\alpha(R). \qquad [6]$$

$g(R)$ is the solute–solvent radial pair correlation function, V is the volume of the system, and N the number of solvent molecules. $N\langle\nu^2(R)\rangle$ is the square of the inhomogeneous width Γ of the absorption band considered which is centered at a frequency ν_0. The frequency where hole burning is performed is ν_b. Since this frequency can be selected by tuning the burning laser over the inhomogeneous band, it is a parameter of the experiment.

The specific features of the system are reflected in the perturbation $\nu(R)$. However, some important conclusions can be made without considering the details of the interaction. The critical quantity in this context is the degree of correlation, Eq. 5, which can, in principle, be determined from the experimental results.

We stress that ρ is an independent quantity, depending on the structural properties of the system solely, since the correlation between two quantities (α and ν) is basically independent of their square averages. Let us consider the case $\rho \to 1$. Then, $\alpha(R) \sim \nu(R)$. From Eq. 2, it is obvious that this case implies $\nu(R) \sim 1/R^n$. Then Eq. 3 becomes

$$S = \frac{n\kappa}{3}(\nu_b - \nu_{vac})\Delta p. \qquad [7]$$

A reasonable choice for n would be 6, as is the case for dispersion or higher-order electrostatic interactions. Since $\nu_b - \nu_{vac}$ is the solvent shift, Eq. 7 states that pressure shift and solvent shift are proportional (26).

With Eq. 7, it would be possible to measure directly the vacuum frequency, ν_{vac}, of the chromophore and the compressibility of the solvent. Note that in reality a protein is not isotropic. Hence, the compressibility has tensorial character. However, our experiment averages over the spatial variations. The important question then is whether $\rho \approx 1$ reflects a realistic situation. $\rho \to 1$ would imply that there were no pressure broadening; hence, the obvious conclusion would be that $\rho \to 1$ is unrealistic. However, from all that we know from numerous experiments on glasses (27–30), this is not the case. If $\rho = 1$ is possible, where does the broadening σ come from? We stress that ρ influences σ in a quadratic fashion. Even small deviations from 1 can lead to a significant broadening. Moreover, the dispersion-type forces have angular degrees of freedom which can lead to a broadening. Note that these angular degrees of freedom have no influence on Eq. 7 and the basic assumptions involved—namely, $\nu(R) \sim R^{-n}$—because we average over them. Hence, they just contribute to a scaling factor. The conclusion is that a pressure-induced broadening does not necessarily contradict a high correlation between α and ν in Eq. 3 and, hence, Eq. 7 seems to describe the situation reasonably well.

Previous studies measured compressibilities by using the hole-burning technique to study MP-HRP at pH 5 (22), protoprophyrin-substituted myoglobin (24), and a series of alcohol glasses doped with several dyes (27, 29–31). We stress that the results obtained fit well into the scenario known from other experiments, such as sound velocity measurements (32), Brillouin scattering (33), and mechanical experiments (34).

Experimental Procedures

Sample Preparation. Isoenzyme C2 of HRP was isolated and purified by K.-G. Paul, as described (35), and kindly donated to us. The apoprotein was prepared as described (36) and was reconstituted with purified mesoporphyrin IX (37). The reconstituted protein was stored at 200 K in 50 mM ammonium acetate at pH 5. As needed, the pH was adjusted to pH 8 by adding K_2HPO_4, and the environment of the protein was changed by dialysis. For optical spectroscopy, 20–40 μM protein samples were used and 50% (vol/vol) glycerol was added to ensure transparency at low temperatures.

Spectroscopy. The samples were sealed in small plastic bags to ensure isotropic pressure conditions. Pressure was transmitted via helium gas. The pressure cell was immersed in liquid helium and kept at a temperature of 1.5 K. The pressure level was accurate within 1 kPa. Holes were burned with a ring dye laser pumped by an argon ion laser. The bandwidth of the laser was on the order of 10^{-4} cm^{-1}. To obtain a sufficiently good signal/noise ratio, the holes were burnt to a relative depth of 20–40%. Detection was made by scanning the dye laser over the hole and observing the fluorescence emission.

Broad-band photochemistry was performed with the ring dye laser, which was set to various frequencies within the B1

band and was continuously scanned over 30 GHz. The average power level was 10 mW and irradiation times were on the order of 1 min.

Results

Fig. 1*a* shows the fluorescence excitation spectrum of MP-HRP at pH 8 after sudden freezing from room temperature to 4.2 K. The spectrum appears similar to the respective one at pH 5 (22). Our work is focused on the origin region between 16,000 and 16,400 cm^{-1}. It is characterized by two prominent bands, B1 and B3. The two bands correspond with different tautomer states of the chromophore, as is immediately obvious from the photochemical behavior (Fig. 1 *b* and *c*): irradiation into the B1 band produces an additional band (B2) but affects B3 only little. Hence, B1 and B3 are largely decoupled. B2 corresponds with additional tautomer states, which are only very weakly populated in the original sample (Fig. 1 *a* and *b*).

Fig. 2*a* shows a hole burnt into the photochemically enriched B2 band. As pressure is increased, the hole clearly splits into two components. Both of them experience a red shift, but of different magnitudes. Fig. 2*b* summarizes the pressure shift data of Fig. 2*a*. Two facts are demonstrated: (*i*) the shift is, for both components, perfectly linear with pressure and (*ii*) the slopes are significantly different.

The data in Fig. 3 pertain to the unbleached sample. The shift per pressure as a function of burn frequency for the entire origin region is shown. (*i*) There are linear regions (filled symbols), in accord with Eqs. **3** and **7**. (*ii*) In between

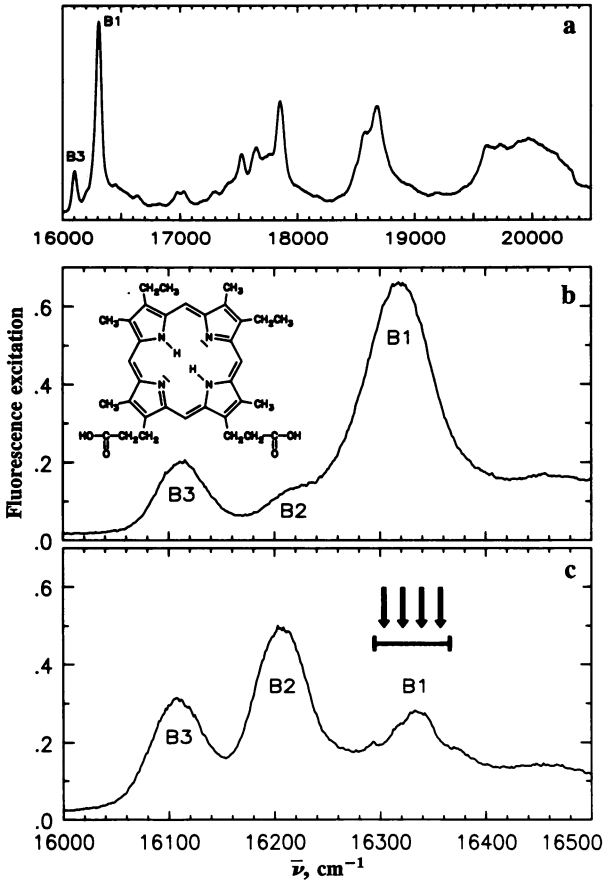

FIG. 1. (*a*) Overview of the spectrum of MP-HRP at pH 8. Temperature was 4.2 K. (*b*) Spectral range of the origin on an enlarged scale. *Inset* shows the structure of MP. (*c*) Photochemically induced changes of the origin range. Arrows indicate the spectral range where light irradiation occurred. Units on the ordinate in *b* and *c* are arbitrary.

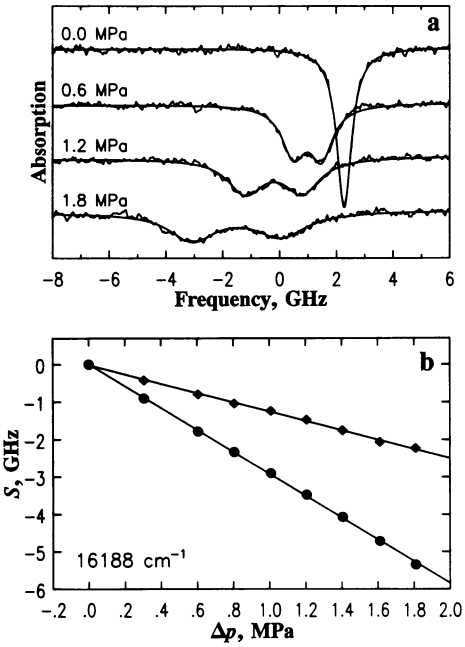

FIG. 2. (*a*) A hole burnt into band B2 at 16,188 cm^{-1} and its changes under increasing pressure. Δp values are indicated. (*b*) Pressure shift (*S*) of the two components of the hole in *a*.

the bands, significant deviations from linearity occur. (*iii*) In these nonlinear ranges is the onset of a bifurcation of the pressure shift; the holes split. (*iv*) The slope of the linear range in the B1 band is quite different from that in the B2 and B3 bands.

Fig. 4 shows the same experimental series as Fig. 3, but after extensive photochemical bleaching of band B1. Band B2 is now the most prominent one. Hence, the data in the B2 region have higher significance. Basically, the results are the same: the slopes in the B3 and B2 ranges differ a little; however, the fact that they are almost a factor of 3 larger than that of the B1 band is confirmed.

According to Eq. **7**, the measured slopes are related to the compressibility of the protein. Below, we will explain why these slopes can be different, why ranges with nonlinear behavior can be present, and why holes split. As we will see, these features seem to be characteristic for proteins. They point to specific properties of their structural phase space.

Discussion

Discrete Site Splitting Under Pressure and Nonlinear Frequency Dependence. Let us assume that two different tautomer species with different vacuum absorption frequencies

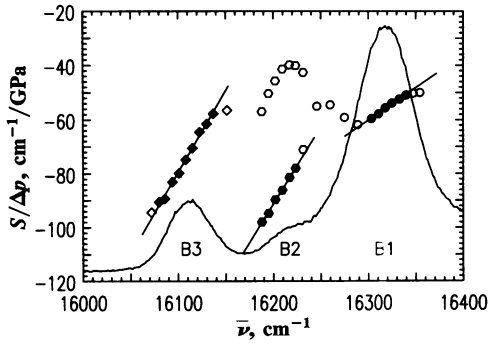

FIG. 3. Lineshift per unit pressure change as a function of burn frequency prior to photochemical treatment of the sample. The filled symbols were used for data evaluation.

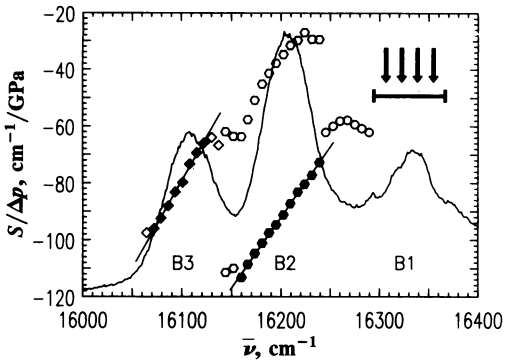

FIG. 4. Lineshift per unit pressure change after photochemical enrichment of tautomer B2. The filled symbols were used for data evaluation.

overlap with their inhomogeneous envelope in a certain frequency range. Hole burning in this range will affect both tautomers. When pressure is changed, the hole will broaden and shift. However, according to Eq. 7, the shift will be different for the two species, since they have different vacuum frequencies. These different shifts lead to a splitting Δ_s. From Eq. 7, we get

$$\Delta_s = 2\kappa|\nu_{vac,1} - \nu_{vac,2}|\Delta p. \quad [8]$$

Here, we have assumed that the environment of the chromophores is characterized by a well-defined compressibility. We will relax this assumption below. From Eq. 8, it is obvious that the splitting Δ_s is frequency independent. For our system, this holds only approximately (Fig. 4).

The data also convincingly demonstrate that the splitting is not due to an overlap of bands B1 and B2. Instead, the B2 band originates from two different tautomers. Otherwise, the slope of the lower branch of the two components should coincide with that measured in the B1 band. Hence, all in all, the origin region investigated consists of at least four tautomer states (20).

Whether a splitting can be observed or not depends on the relative magnitudes of the shift and the concomitant pressure broadening. If the latter is larger, the two components cannot be resolved and an average shift and broadening will be observed. This gives rise to nonlinearities in the frequency dependence. Also, at the band edges, the weight of one component becomes small compared with the other. A splitting cannot be measured anymore. However, the low-weight component still affects the behavior of the hole under pressure, leading to asymmetric lineshapes. In this case, strong deviations from a linear color effect occur as well.

Correlated Phase-Space Model. We will discuss the specific pressure phenomena observed—namely, a compressibility which varies with the excitation frequency—on the basis of a model which we call the correlated phase-space model. We want to sketch the basic aspects of the model first.

Let us consider the structural states of the chromophore and the apoprotein. The number of states for the chromophore is small whereas the number for the apoprotein is huge. Previous studies have shown that MP in HRP has at least four different pyrrole tautomers. They can be switched from one form to the other by light irradiation or by heat treatment (20).

If there is no correlation between the tautomer states of the chromophore and the conformational substates of the protein, the compressibility, as measured by the hole-burning technique, will reflect an average value over all protein conformations and will not change if the chromophore is switched to another configuration. In the correlated phase-space model, however, we assume that specific structures of the chromophore correlate with specific structures of the protein. Within the frame of this model, switching from one tautomeric form to another would select another area in the phase space of the apoprotein, as long as the apoprotein could accommodate its structure. As a consequence, any structure-sensitive parameter—e.g., the compressibility—should depend specifically on the selected tautomer. The reverse of this argument has been tested in our previous work, where we induced significant structural changes of the protein by binding an aromatic molecule near the heme. This led to different tautomeric configurations, and the compressibility changed by a factor of 3 (23).

Correlation is supposed to prevail as long as the relevant energy scales are of the same order of magnitude. In our system, the energies to be compared are the mean amplitude of the energy fluctuations in the protein, as reflected in the inhomogeneous width of the spectral bands, and the energy differences between the tautomeric forms. It is obvious that the protein needs structural freedom to accommodate its conformation to the configuration of the chromophore. Hence, it can happen that such an accommodation takes place at room temperature but is suppressed at low temperatures.

Specific Pressure Phenomena in the Various Tautomer Bands. In the following, we use the correlated phase-space model to explain the tautomer-specific features in our results. According to Eq. 7, the slope of the frequency shift per pressure is determined by the compressibility of the host material. In our case, the host material is the protein. The problem we have is that there are definitely two significantly different slopes (Figs. 3 and 4) and, hence, two different compressibilities, 0.12 and 0.3 GPa^{-1}, respectively. The compressibilities measured in the tautomer bands B3 and B2 are almost a factor of 3 larger than the compressibility measured in the B1 band. We found a similar behavior for MP-HRP at pH 5 and for protoporphyrin-substituted myoglobin (38). It seems that such a behavior is specific for proteins. For instance, when alcohol glass was measured with several quite different dye probes, there was no dye specific difference in the respective slopes (27). The conclusion is that, although proteins have very much in common with glasses, they are different. In a glass, there are no distinguished structural states. Although a glass is nonergodic, its structural phase space is homogeneous. Consequently, ensemble averages are characteristic for the material and have definite magnitudes. In a protein, it seems that there are distinguished structural states. In myoglobin, for example, the so-called taxonomic states fall into this category (9). If the protein is frozen, it will be trapped in conformational substates. Conformational substates with smaller structural variations group around the distinguished states. In other words, the protein occupies certain islands in conformational phase space. It is even conceivable that these islands themselves are built from smaller islands so that the phase space shows a kind of self-similarity. At sufficiently low temperatures, the islands are decoupled. Hence, as compared with glasses, proteins reflect additional inhomogeneities. They can exist in different states which can eventually be distinguished in a macroscopic way.

This is at the heart of the correlated phase-space model. The islands correspond with specific tautomer states which can be optically selected. Because of this selection, the respective ensemble averages are not anymore averages that comprise the whole phase space. Instead, they are averages over the respective islands only. Consequently, the compressibility which we measure at a certain tautomer band reflects the compressibility of the protein in the associated island state. The remarkable thing is that these compressibilities can be drastically different. There is another interesting fact: the slope in the B2 band shows almost no change in going from the original sample to the sample with a photochemically en-

hanced B2 tautomer. From this observation we have to conclude that the HRP apoprotein changes its structure during phototransformation of its chromophore despite the fact that it is frozen in a glass at 4.2 K. This is a clear indication that there are, in addition to large structural barriers, many low barriers which can easily be overcome. This observation is in line with recent spectral diffusion experiments on HRP which showed that some of these structural barriers have energies as low as a few wavenumbers (39).

We argued in the preceding paragraph that the B2 band is built from a pair of tautomer states. According to the correlated phase-space model, this pair of tautomer states may be connected with different protein structures and, hence, with different compressibility values. If so, the pressure splitting of the hole should not be constant but should depend on burn frequency. From Eq. 7 we get, in this case,

$$\Delta_s = [2|\kappa_1 - \kappa_2|\nu_b - 2(\kappa_1\nu_{vac,1} - \kappa_2\nu_{vac,2})]\Delta p. \quad [9]$$

A closer look to Figs. 3 and 4 reveals that the data can indeed be interpreted this way. The slope of the open symbols is different from that of the filled ones. It should be stressed, however, that close to the bifurcation points the holes are difficult to evaluate and, since the inhomogeneous width of the tautomer bands is not too large, the number of data points in a range with constant slope is rather low. Hence, the quantitative aspect should not be overstressed. All we want to say is that the data do not contradict this view.

This statement brings us to a final comment on the accuracy and significance of our pressure-tuning experiment. Since there are several tautomer states, overlap regions with nonlinear frequency dependence occur, and hence it is important to address the significance and accuracy of the data. From Fig. 2, it is evident that the shift is linear with pressure. Hence, the associated slopes, which are the quantities of interest, are generally very accurate. Of course, close to the bifurcation points, problems occur. For instance, in Fig. 3, the data points in the neighborhood of the high-energy bifurcation point tend to increase the slope of the lower branch of B2. This effect is less significant in Fig. 4, where the spectral weight of the B2 tautomers is enhanced by more than an order of magnitude. Hence, the slope is lower there by roughly 15%. Since the slope in the B3 band is not affected through photochemical transformation, the B3 and B2 tautomers show a tendency toward different compressibilities, in agreement with the correlated phase-space model. Again the small variations in the slopes should not be overestimated as far as quantitative conclusions are drawn. On the other hand, a change by a factor of almost 3 in going from the B1 to the B2 and B3 tautomers is a significant result. It has been proven by many experiments with different samples. It is supported by similar experiments on the same protein at pH 5, where similar changes in a different set of bands were observed. Hence, this result is firm and we think it fits beautifully into the scenario known from other work on proteins.

Summary

Pressure tuning hole burning experiments on a MP-HRP protein over the entire frequency range of the origin multiplet revealed interesting features. There are at least four tautomeric states. One tautomer is degenerate. The associated holes split into a doublet under pressure. This splitting leads to bifurcation points in the frequency dependence of the pressure-induced line shift. In the main ranges of the bands, this frequency dependence is linear and can be used to determine the compressibility of the protein. The remarkable observation is that the compressibilities measured at various tautomer bands can differ by as much as a factor of 3. This behavior can be interpreted on the basis of the so-called correlated phase-space model, which assumes that the protein accommodates its structure to the tautomer configuration (or vice versa), thereby inducing a change in its structural parameters, including the compressibility.

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