Softening of the packing density of horseradish peroxidase by a H-donor bound near the heme pocket

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ABSTRACT We use pressure tuning of spectral holes to estimate the compressibility of protein molecules by optical means. We found that the compressibility of mesoporphyrin-substituted horseradish peroxidase increases by a factor of three when it incorporates small aromatic H-donor molecules that bind in the vicinity of its heme pocket. Such a dramatic softening of its packing density corresponds to a jump from a compressibility range characteristic for the solid state into that characteristic for liquids.

INTRODUCTION

Horseradish peroxidase (HRP) is a single-chain heme protein that catalyzes the decomposition of H_2O_2 in the presence of H-donors bound in the neighborhood of the heme (1). Details of this two-step reaction have been studied extensively; however, the absence of x-ray crystallographic data for this enzyme hampers the clear insight into the structural changes involved. Thus, considerable emphasis has been placed on spectroscopic techniques and computer simulations. As a reference, cytochrome *c* peroxidase, a molecule with similar size and enzymatic function and with known x-ray crystallographic structure, generally has been used (2).

In this article, we report on spectral hole-burning experiments performed on HRP under conditions where the enzyme binds an aromatic H-donor-molecule (4–6). The special feature of our technique is that we can investigate the behavior of the burned-in holes under isotropic pressure conditions (7). A change in pressure leads to a shift and to a broadening of the holes that depend on the solvent shift of the chromophor and its protein environment (8, 9). Since the environment is inhomogeneous, the solvent shift varies when burning the holes at various positions in the inhomogeneous band profile. From the pressure shift as a function of burn frequency, it is possible to determine in a straightforward way the compressibility of the protein molecule, provided it can be treated as an elastic and homogeneous medium on the scale of the relevant interaction (10). Burned-in holes can be extremely narrow; hence, the technique is so sensitive that significant line shifts occur at pressure changes on the order of 0.1 MPa. We could verify that under such low-pressure conditions, proteins behave as fully elastic solids (10, 11).

The compressibility of a protein molecule is a macroscopic parameter in the sense that it characterizes an overall property of the biopolymer. As we will show, the inclusion of a rather small aromatic substrate molecule in the heme pocket leads to a dramatic change of the compressibility. The packing density softens in a way that the protein appears to be more liquid-like than solid-like. It is this aspect that we want to stress in this article. It seems probable that the biological function in the presence of an aromatic H-donor in the pocket is based on a significant structural change within the whole protein.

Characterization of HRP under substrate binding conditions

Results concerning the binding of aromatic H-donors (substrates) to native HRP indicate a 1:1 stoichiometry and the involvement of hydrophobic interactions with an aromatic amino acid residue in the pocket (4–6) at a distance of 5.8–11.2 Å from the heme iron (12, 13). The importance of hydrogen bonding at the binding place also has been emphasized (4, 5). There have been contradictory interpretations concerning the identification of the binding place: whether or not the bound substrate is coordinated to the iron of the heme (14, 15) or the sixth coordination place will be occupied by a water molecule when the substrate is bound (15). Recent results, however, seem to support the model already suggested in the earlier articles that the various aromatic H-donors bind at the same location within the pocket, close to the 8-CH_3 group of the heme and to Tyr-185 and Arg-183 of the protein, i.e., at the edge of the porphyrin ring, with a molecular plane almost perpendicular to the porphyrin plane (15). Also, our own studies (16) show no evidence for a sandwhich-type binding between NHA and MP. (A schematic sketch [2, 17] of HRP with the substrate molecule attached to the heme is shown in Fig. 1. The relative scale has the correct order of magnitude.)

Basic aspects of pressure effects in spectral hole burning as applied to proteins

Experimental evidence has been published indicating that the behavior of proteins at low temperatures can, in many cases, be understood on the basis of glass models (18–20). However, in some parameters, the special order and finite size of a protein can lead to specific deviations from glassy behavior (21, 22).

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In recent years, theoretical models of chromophores embedded in glassy matrices have been published that describe inhomogeneous broadening effects on spectral lines as seen in hole-burning experiments (8, 9, 23). In the present article, we want to focus on the line shift of a spectral hole as affected by pressure (9). A nonergodic system, like a protein, can suffer irreversible conformational changes under sufficiently high pressure changes (24). However, it has been shown experimentally that under the low pressure conditions of our experiment, proteins show perfectly reversible behavior (10, 11) and, hence, can be treated as an elastic solid.

In the following, we consider a chromophore in an isotropic and homogeneous, yet disordered, environment. It is our goal to find out how its transition frequency changes when the sample is compressed. Suppose the chromophore, when completely isolated, absorbs light at a frequency ν_{vac}, the so-called vacuum absorption frequency. When put into an interacting environment, a solvent molecule (or in the case of a protein, the constituent atomic groups) at a distance R may change this frequency by an amount $\nu(R)$. If pressure is applied, then $\nu(R)$ will change by an additional amount $\Delta\nu(R)$. In the low-pressure range of our experiment, a first-order expansion of $\nu(R,\Delta p)$ with respect to Δp is sufficient, and we get

$$\Delta\nu(R) = \frac{\delta\nu(R)}{\delta R}\frac{\delta R}{\delta p}\Delta p. \\ := \alpha(R)\Delta p. \quad (1)$$

Since we had assumed that the environment is isotropic and homogeneous, $\delta R/\delta p$ is given by

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

where κ is the homogeneous, isotropic compressibility. The details of the model appear in the chromophore-solvent interaction, i.e., in the functional form of $\nu(R)$. Laird and Skinner (9) have shown recently that a modified Lennard-Jones potential is necessary to describe the pressure induced broadening of spectral holes. However, as far as the line shift effects are concerned, it seems sufficient to consider the attractive part of the Lennard-Jones potential only. This part falls off $\propto 1/R^6$. Its interaction range is much larger as compared with the repulsive part of the potential. With this approximation, Eq. 1 and 2 lead to

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

In a real experiment, when burning a hole, we see the contributions from all the chromophores with transition frequencies identical to that of the laser frequency. That is, we have to average Eq. 3 over all chromophore-matrix configurations with identical absorption frequencies to get the measured pressure induced frequency shift s:

$$s = 2\kappa\langle\nu(R)\rangle_c\Delta p. \quad (4)$$

$\langle\nu(R)\rangle_c$ is the solvent shift of those molecules, which have been selected by the burning laser. The suffix c indicates that the averaging procedure involves selected configurations. Since $\langle\nu(R)\rangle_c$ is the solvent shift of the frequency selected molecules, it is related, by definition, to the vacuum absorption frequency of the chromophore via

$$\langle\nu(R)\rangle_c \equiv \nu_b - \nu_{vac}, \quad (5)$$

where ν_b is the burning frequency. With definition (5), Eq. 4 becomes:

$$s = 2\kappa(\nu_b - \nu_{vac})\Delta p. \quad (6)$$

Note that according to this formula, the shift depends linearly on the burn frequency ν_b. By tuning the frequency of the burning laser over the inhomogeneous band, this linear dependence can be measured. Then, from a plot of the shift per unit pressure against the burn frequency, the compressibility and the average solvent shift ν_s, defined as the difference between the maximum position of the inhomogeneous band and the vacuum frequency, also can be determined (7, 8, 11).

In this model description, there are three important approximations involved: (*a*) the matrix is homogeneous and isotropic; (*b*) the solvent molecules are assumed to be decoupled from each other and to interact with the chromophore only; and (*c*) the pressure-induced shift is proportional to the solvent shift. This ansatz implies a restriction to a R^{-n} – type potential, e.g., a dispersion type interaction ($n = 6$).

Point 3 seems to be the most severe approximation. If one abandons the proportionality between solvent shift and pressure shift, one ends up with the more general Laird-Skinner-model (9):

$$s = \left[N\langle\alpha\rangle + \frac{\langle\alpha\nu\rangle}{\langle\nu^2\rangle}(\nu_b - \nu_0)\right]\Delta p, \quad (7)$$

where ν_0 is the maximum of the inhomogeneous band and N is the number of solvent atomic groups involved. The quantities in brackets are averages over all possible configurations as expressed by the respective integrals over the two particle correlation function $g(R)$, for instance,

$$\langle\alpha\rangle = \frac{1}{V}\int \alpha(R)g(R)\,dR, \quad (8)$$

where V is the volume.

The term $1/Vg(R)$ can be interpreted as the probability of finding a solvent molecule at a distance R from the dye probe. The term $\alpha(R)$ is defined in Eq. 1. It represents the change in the interaction between the probe and a solvent molecule at a distance R, per unit pressure. Eq. 7 holds for $\nu(R)$ caused by any kind of interaction. Note that Eq. 7 still shows a linear dependence on burn

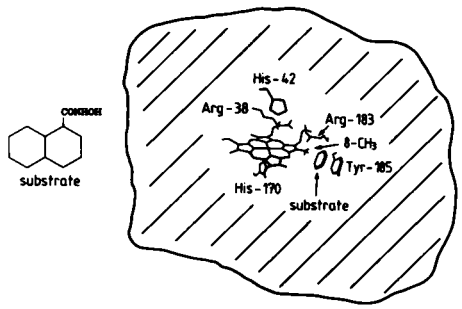

FIGURE 1 Sketch of the heme crevice of HRP with a substrate molecule (naphthohydroxamic acid) at its binding place. The structure is taken from Teraoka and Kitagawa (15). Note that the sketch reflects the relative dimensions on a correct scale.

frequency ν_b. However, the slope factor (Eq. 6) is not any more dependent on the compressibility κ solely, but scales in addition with the degree of correlation ρ between $\alpha(R)$ and $\nu(R)$. ρ is defined by

$$\rho^2 = \frac{\langle \alpha\nu \rangle^2}{\langle \alpha^2 \rangle \langle \nu^2 \rangle}, \quad |\rho| \leq 1. \tag{9}$$

In principle, ρ can be determined experimentally by measuring not only the pressure-induced shift but also the concomitant broadening β:

$$\beta^2 = N\langle \alpha^2 \rangle (1 - \rho^2) \Delta p^2. \tag{10}$$

Note that $N\langle \nu^2 \rangle$ is the inhomogeneous width. Once ρ is known, the parameters ν_s and ν_{vac} can be evaluated without any specific assumption on the potential used.

As for κ, one still has to use model potentials. We stress that in the above model, κ is a local compressibility. It characterizes the material within the radius of the interaction potential. Our experiments demonstrate that this range does not exceed the size of the protein studied.

We conclude this section by stressing that cross-checks of the determined parameters are possible. We will show that the compressibilities of proteins determined by relation 6 fall well into the range determined by other techniques, e.g., sound velocity measurements. This agreement suggests that the simple line shift model is most probably good enough to extract parameters of the correct order of magnitude.

MATERIALS AND METHODS

For the measurements, the native iron proto-heme of HRP has been replaced by free-base mesoporphyrin (MP) to make the sample fluorescent and to get the advantage of having an excellent chromophore for photochemical hole burning (10, 21, 22, 25). It has been shown earlier (5, 16, 26) that aromatic H-donors bind to HRP with high affinity even when reconstituted with a metal-free porphyrin. As a representative of aromatic substrates, naphthohydroxamic acid (NHA) (see Fig. 1) has been chosen because of its high binding affinity among the H-donors studied (5, 27).

HRP isoenzyme C2 has been isolated from horseradish roots and purified as described by Paul and Stigbrand (28). The pure fraction was treated with 2-butanone (29), and the apoprotein was recombined with purified mesoporphyrin IX. The sample was generously offered for our experiments by K.-G. Paul. NHA was synthesized by G. R. Schonbaum (St. Jude Children's Research Hospital, Memphis, TN) and kindly donated to us. Samples were prepared in 50 μM ammonium acetate, pH 5, and 50% glycerol was added to assure transparency. The concentration of MP-HRP was 20 μM, and NHA was added up to a molar ratio of 5:1 related to HRP.

Spectral holes have been burnt into the fluorescence excitation spectrum at 1.5 K to a relative depth of 50%. The laser source for hole burning was a single-mode continuous wave (CW) dye laser (model 699-21; Coherent, Inc., Palo Alto, CA) characterized by a bandwidth of the order of 10^{-4} wavenumbers. The power levels used for burning were in the microwatt range. Spectral holes were measured by scanning the laser over the burn frequency. The overall scan range was 1 wavenumber. The intensity of the detection beam was reduced by two orders of magnitude as compared with the burning beam. Pressure has been applied isotropically through He gas and could be measured within an accuracy of 10 hPa. Typically, it was varied in increments of 0.2 MPa. The upper limit of pressure in this experiment was determined by the solidification of liquid He at 2.5 MPa, at the temperature of this experiment. To assure isotropic pressure conditions, the sample has been sealed in a plastic bag when immersed in liquid He.

RESULTS

In Fig. 2, a low temperature (1.5 K) fluorescence excitation spectrum of MP-HRP is shown with a resolution of 5 cm^{-1}. A previous study (30) has shown that the sharp peaks, an intense peak at 16,310 cm^{-1} (labeled by 1), and a small peak at 16,090 cm^{-1}, respectively, are (0,0) bands of different tautomeric forms of free-base MP. Adding NHA to the protein (spectra in Fig. 2, *b–d*) leads to the appearance of new species characterized by two new (0,0) bands at 16,180 cm^{-1} (labeled by 3) and at 16,260 cm^{-1} (labeled by 2), respectively. The binding process saturates around a molar ratio of 2.5.

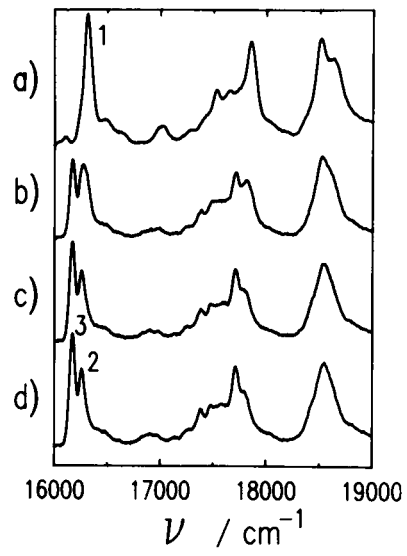

FIGURE 2 Complex formation of HRP and NHA as reflected in the fluorescence excitation spectrum. The spectra were measured at 1.5 K in a glycerol/water solution. The ratio of concentration of NHA molecules and HRP molecules is as follows: (*a*) no NHA, (*b*) 1 ÷ 1, (*c*) 2.5 ÷ 1, and (*d*) 5 ÷ 1.

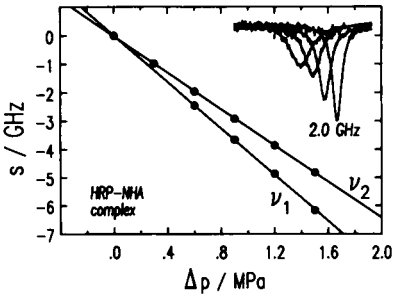

FIGURE 3 The inset shows how a hole, burnt into the absorption of the HRP-NHA complex, changes under pressure. The two straight lines reflect the frequency shift of the burnt-in holes with pressure for two burn frequencies: ν_1 = 16,159 cm^{-1} and ν_2 = 16,195 cm^{-1}. Temperature, 1.5 K.

Fig. 3 demonstrates the influence of pressure on a spectral hole burnt into the 16,180 cm^{-1} band of the MP-HRP/NHA complex. The inset shows how the hole changes its shape, its spectral position, and its width under increasing pressure.

A plot of the hole shift versus pressure change shows the linear dependence of s on pressure according to Eq. 6. It is also demonstrated that the slope of these lines depends on the burn frequency (ν_1, ν_2).

In Fig. 4 *a*, we show the shift s per unit pressure as a function of the burn frequency ν_b within the inhomogeneous band. The sample is the HRP-NHA complex. As can be seen, the experimental points fall perfectly onto a straight line, in agreement with Eq. 6. We take this as strong evidence that the model used is sufficiently accurate to describe the pressure effects on spectral holes in proteins.

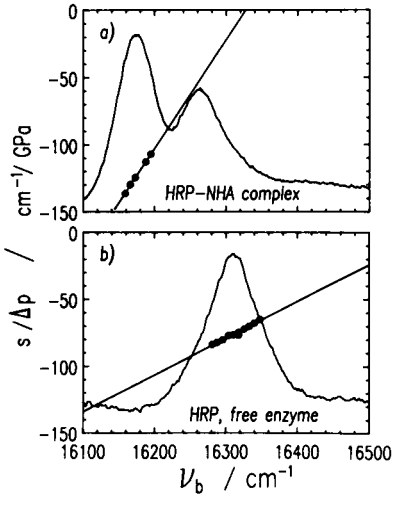

FIGURE 4 (*a*) Frequency shift of a hole per unit pressure ($s/\Delta p$) as a function of burn frequency within the inhomogeneous band of the HRP-NHA complex. The two tautomer bands are also seen in Fig. 2 *d*. (*b*) Frequency shift per unit pressure for the free enzyme (see Fig. 2 *a*). Note that the two slopes differ by a factor of 3.

TABLE 1 **Comparison of various parameters of the free MP-HRP and the complex with NHA**

	κ	ν_0	ν_s	ν_{vac}	$\Delta V/V$
	GPa^{-1}	cm^{-1}	cm^{-1}	cm^{-1}	%
MP-HRP (band 1)	0.14	16,310	280	16,590	0.2
MP-HRP/NHA (band 3)	0.4	16,180	150	16,330	0.4

κ, compressibility; ν_0, maximum of the inhomogeneous band where hole burning was performed; ν_s, average solvent shift; ν_{vac}, vacuum absorption frequency of the chromophore; $\Delta V/V$, equilibrium volume fluctuations.

For comparison, data for the free enzyme MP-HRP are shown in Fig. 4 *b*. In this case, the pressure tuning experiment has been performed within the most intense tautomer band of the free enzyme around 16,310 cm^{-1} (see Fig. 2 *a*). A striking difference between the slopes of the $s/\Delta p$ versus ν_b plots for the free and complexed protein is evident. Since, according to Eq. 6, this slope is determined by the isothermal compressibility κ, we conclude that the incorporation of the substrate molecule NHA into the protein changes the compressibility of HRP from 0.14 to 0.4 GPa^{-1}, i.e., roughly by a factor of 3.

From the compressibility, the equilibrium volume fluctuations of the protein can be determined:

$$\frac{\Delta V}{V} = \sqrt{\frac{\kappa k_B T}{V}}. \qquad (11)$$

The volume of HRP can be estimated by comparison with myoglobin. It is on the order of 10^5 $Å^3$. Then, we estimate the complexed enzyme volume fluctuations at ambient temperatures to be on the order of 0.4%. This is almost a factor of 2 larger than for the free enzyme. Note that these are lower estimates since κ may change as the temperature is increased from 1.5 K to room temperature.

The relevant parameters that can be determined from the pressure-tuning hole-burning experiment are summarized in Table 1. These are the isothermal compressibility κ, the average solvent shift ν_s, the vacuum frequencies ν_{vac} of the chromophore(s), and the respective volume fluctuations for the free and the complexed enzyme. ν_0 is the maximum of the band examined by the hole-burning experiment.

Note that the chromophore in the complex has a different vacuum frequency than in the free enzyme.

DISCUSSION

Comparison with frequency selected fluorescence spectra

It has been shown (21, 30) that the chromophore in free HRP can be reversibly transformed, photochemically or

thermally, into at least four different tautomeric forms separated by (0,0) energies on the order of 100 cm^{-1}. As can be seen from Fig. 2, the spectrum, characteristic for the complex of the protein with NHA, has two distinct (0,0) maxima without photochemical treatment.

The results, presented in Fig. 2, are in agreement with our previous studies on complex formation of MP-HRP and NHA (27) using fluorescence line-narrowing (FLN) techniques. Since for these kinds of experiments usually much higher intensities are required as compared with hole-burning experiments, the FLN-spectra were representative of photobleached samples. The main difference between these FLN results and the present hole-burning spectra is that, instead of two new bands (2 and 3, Fig. 2), only one could be seen. From this comparison, it follows that band 2 and 3 represent two tautomeric forms that can be converted under irradiation. As for the overall change in the (0,0) range shown in Fig. 2, it seems to be clear that the binding of the substrate causes a red shift and decreases the tautomer splitting.

Proteins as glasses: how good is the model?

The evaluation of the data as shown in Figs. 3 and 4 was based on Eq. 6. The most important question in context with pressure-tuning hole-burning spectroscopy is, of course, whether or not this simple model can be applied to proteins.

Generally speaking, on a qualitative basis, the model works perfectly, as is most obvious from the linear color effect of the lineshift per unit pressure shown in Fig. 4. The model has two parameters, namely the vacuum frequency, ν_{vac} (or, equivalently, the average solvent shift ν_s), and the compressibility κ. We have to ask what the numbers, which we determine from the experiment, really mean.

The critical quantity in this context is the degree of correlation ρ between pressure shift and solvent shift (Eq. 9). If the correlation were perfect ($\rho = 1$), the above model would hold. However, in this case, there would not be any pressure-induced broadening of the hole (Eq. 10), which is not the case. On the other hand, if correlation were totally absent ($\rho = 0$), there would be broadening but no color effect in the shift. The degree of correlation is, in principle, experimentally accessible. We measured recently a series of alcohol glasses and found ρ to be rather high (0.75). Hence, for these systems, the errors in the above parameters κ and ν_s due to the simplified model are on the order of 25–30%. This is in agreement with independent experiments.

The degree of correlation is determined by the differential potential $v(R)$. A high correlation implies that $v(R)$ scales like R^{-n}. But even for a pure dispersive interaction ($n = 6$), correlation is not perfect, because there are angular degrees of freedom that lower ρ.

In proteins, the situation is more complicated. First, the measured ρ is lower than in the glasses investigated, which means that deviations from the true values can be larger. Second, the variations in the system parameters may be larger. Variations may arise from the fact that, on sufficiently short-length scales, proteins are neither isotropic nor homogeneous. If, for example, the intermolecular interaction responsible for the pressure shift were significantly influenced by the repulsive part of the Lenard-Jones interaction, then we would expect different compressibilities, when the experiment is performed in bands of different tautomer forms of the chomophor. Different tautomers may have different interactions with their nearby environments. The point is that these differences are not averaged to zero due to the lack of randomness on sufficiently short-length scales. The variations in the parameters are currently investigated.

If, on the other hand, the relevant chromophore-environment interaction occurs on a sufficiently large scale, these variations are not significant and the protein can be described sufficiently well as an isotropic and homogeneous solvent for the chromophore.

As stressed above, ν_s and κ could be determined with sufficiently high accuracy from Eq. 6 and the degree of correlation. If we evaluate ρ from our experiments using Eq. 7 and the line broadening under pressure (Eq. 10), we find $\rho \approx 0.3$. Such a low ρ would imply that the parameters are uncertain within a factor of 3. However, there is one big problem that hampers a correct evaluation of ρ. The reason is that there are interactions that lead to a broadening but not to a line shift. For example, the chromophore in HRP is polar and the solvent is polar. Hence, there is a dipole–dipole (and even an ion–dipole) interaction between the randomly oriented solvent molecules and the chromophore that is of infinite range and that may contribute significantly to the width β (Eq. 10), and, hence, may decrease ρ. However, since random dipoles cannot contribute to the shift (Eq. 7), they cannot influence the slope factor $\langle \alpha\nu \rangle / \langle \nu^2 \rangle$. The conclusion is that the experimentally observed ρ is always a lower limit of the relevant degree of correlation that determines the slope factor $\langle \alpha\nu \rangle / \langle \nu^2 \rangle$ and the compressibility. Note here that a lower correlation means larger disorder. (In organic crystals, ρ is very close to 1.) Hence, comparing again proteins with organic glasses where the experimentally determined correlation was ~0.75, we expect that the relevant degree of correlation in proteins is at least of the same magnitude. In Fig. 5, we show that the parameters determined from the simple relation (Eq. 6) fall very well into the range known from other experiments. This agreement supports the plausibility of our reasoning.

In fact, in this article, it is the ratio of the compressibility parameters of the same protein molecule in the same host glass under two different conditions on which we focus and not so much on their absolute magnitude.

Another important question in this context is the question of how much the solvent outside the protein molecule contributes to the measured compressibility that we

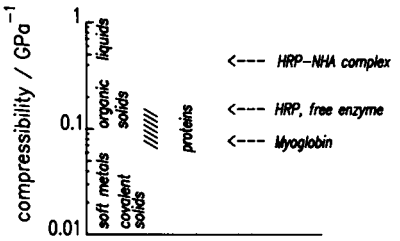

FIGURE 5 Compressibility scale for various materials as taken from a paper by Gavish et al. (36). The protein compressibilities, as measured in our experiments, are indicated.

want to associate solely with the protein. We argued that the pressure-induced linewidth change can be influenced largely by interactions with particles outside of the protein, but the protein is completely decoupled from these as far as the shift is concerned. The large difference in κ found between the complexed and uncomplexed forms of HRP shows that the interaction responsible for the pressure shift (and for the solvent shift) is of sufficiently short range not to exceed the dimensions of the protein. This seems to be the case for the dispersion interaction and other, higher order electrostatic interactions, which are considered as the dominant contribution to the solvent shift (for reviews, see references 31 and 32) and which fall off quickly enough (e.g., as R^{-6}). Indeed, it was shown (33) for myoglobin and hemoglobin that there is a strong dispersion interaction between protein and chromophore that is, for example, largely responsible for the observed rotational strength in these proteins.

CONCLUSIONS

On the basis of these arguments, we assume that the simple model is applicable to proteins. Then, we can evaluate our data according to Eq. 6. From Fig. 4 we learn that $s/\Delta p$ is negative. We conclude that for the complex (Fig. 4 *a*) as well as for the free enzyme (Fig. 4 *b*), the protein environment causes a negative solvent shift of the chromophore absorption. Such a behavior is usually expected for $\pi\pi^*$-transitions in nonpolar solvents. However, the data show that the vacuum frequencies of the chromophore in the complex (band 3) and in the free enzyme (band 1) are different. The same holds for the corresponding solvent shifts; in the complex, this quantity is only half as large as in the free enzyme.

A straightforward explanation of these results is that bands 1 and 3 arise from different tautomers, which experience different solvent shifts through the protein. Obviously, in the presence of the aromatic substrate molecule a different tautomer may be stabilized as compared with the free enzyme. It is also not surprising that the NHA-substrate causes a different solvent shift. However, it should be stressed that NHA influences the inhomogeneous width only slightly. The broadening in the complex and the free enzyme differs only by 30%, respectively. NHA contributes to the average solvent shift only but leaves the inhomogeneous broadening largely unchanged. Consequently, it has no influence on the color effect in the pressure-tuning spectra and, hence, on the compressibility parameter. The latter seems to be solely determined by the apoprotein.

The most striking effect of complex formation occurs in the compressibility. This indicates that the incorporation of the rather small substrate molecule has a dramatic effect on the structure of the protein. In Fig. 5, we represent a compressibility scale (34, 35) for a series of materials as taken from the article by Gavish et al. (36). There, the proteins fall into the range of organic solids. Our results for HRP at pH 5 and myoglobin are indicated and they fit well into the scenery, as has been stressed above.

On incorporation of the substrate molecule, the protein becomes so soft that its compressibility undergoes a jump from the solid-state range to the liquid-state range.

Although we found that the compressibility parameters vary on the order of 20–30% when measured at different tautomer bands, the dramatic change on complex formation is certainly exclusively due to an overall change in the protein structure on incorporation of the substrate. We suggest that the local conformational changes in the heme pocket found by other techniques are going parallel with an overall rearrangement of the apoprotein structure. The interesting question is, what is going on in the protein when it binds NHA near to its pocket? Here we can only speculate. However, from the experiment we can definitely conclude that rather small local perturbations can lead to pronounced global structural changes, in agreement with what has been suggested by Ansari et al. (37).

Related phenomena have been observed in the pressure-dependent spin state equilibria of a series of heme proteins (38–41). Under increasing pressure, the substrate molecule dissociates and, concomittantly, the spin of the central iron atom changes. The dissociation of the substrate is accompanied by a substantial volume change.

In native HRP, at room temperature, the phosphorescence of acetone has been observed that was created at the heme site in an aerobic reaction (42). Fluorescence quenching studies at room temperature in protoporphyrin substituted HRP by molecular oxygen yielded the lowest measured bimolecular quenching rate for O_2 (43). On the basis of these data and of an extensive comparison of various proteins (44–46), the heme crevice of HRP can be considered as a very well-protected volume, surrounded by rigid, organized α-helical protein regions.

We think that the dramatic change from a solid-like to a liquid-like compressibility could occur in the following way: the incorporation of NHA into HRP changes the local electric fields and certainly induces mechanical stress, for instance, by pushing away the Tyr-185 residue (see Fig. 1). The highly organized cage structure releases

this stress by rearranging the organized domains and adopting a new structure. That a thing like this can occur, i.e., that a small perturbation in the cage can lead to a remarkable change in the overall structure, reflects the fact that the energy landscape of a protein is rugged, even at room temperature. A rather small perturbation kicks the whole assembly out of its energy minimum and induces relaxation into a new one. We think that the present experiments together with experiments on spectral diffusion broadening of spectral holes (21, 22) support the idea of a hierarchical structure of the energy landscape of a protein; the spectral diffusion experiments show that the protein (e.g., HRP) can exist in extremely shallow energy minima stabilized by barriers on the order of a few $k_B \cdot T$. Such shallow structural traps are most probably of local origin.

Contrary to these local structural traps, the structural change of HRP induced on substrate binding is kind of a global change, as indicated by the large change of overall properties, such as the compressibility. It seems that such a dramatic softening of the enzyme is of functional relevance. The equilibrium volume fluctuations are increased (roughly by a factor of two) and, consequently, the penetration of small molecules like H_2O_2 to the reaction site becomes much easier. It could well be that HRP undergoes a similar drastic structural change during its enzymatic function as the subunits of hemoglobin do during the oxygen binding process. This latter phenomenon is known as the allosteric effect.

SUMMARY

It seems that the behavior of spectral holes under pressure is well described by a simple model that is based on a high correlation between pressure shift and solvent shift. Within the frame of this model, the compressibility of protein molecules can be determined by optical means. The experiments show that penetration of a small substrate molecule into the pocket of HRP changes the compressibility by a factor of 3. Possible implications of such a behavior on the function of the protein are discussed.

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