Conformational relaxation of a low-temperature protein as probed by photochemical hole burning

Horseradish peroxidase

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ABSTRACT For the first time, conformational relaxation processes have been measured in a small protein, mesoporphyrin-horseradish peroxidase via their influence on spectral diffusion broadening of holes burnt in the fluorescence excitation spectrum of free base mesoporphyrin. Holes were burnt in three 0 ← 0 bands of different tautomeric forms of the chromophore at 1.5 and 4 K, and the spectral diffusion broadening was measured in temperature cycling experiments between 4 and 30 K. The inhomogeneous linewidth for the tautomeric 0 ← 0 bands was estimated to be 60–70 cm^{-1}; the hole width was found narrow, being in the order of 350 MHz (10^{-2} cm^{-1}) at 1.5 K what allowed for an extremely sensitive detection of the conformational changes. Though proteins have many features in common with glasses, the spectral diffusion broadening of photochemical holes under temperature cycling conditions in mesoporphyrin horseradish peroxidase has a very different pattern as a function of temperature. Up to 12 K, the linewidth did not significantly change, then around 14 K, a steplike broadening was observed for all three tautomers, although to a different extent. The total magnitude of line broadening up to 30 K was large and also different for the tautomers. We argue that the difference between the behavior of this protein and that of glassy matrices originate from finite size effects; the protein may be characterized by a small number of TLS, and their distribution may bear discrete features.

INTRODUCTION

The characterization of a protein with respect to known states of matter is in no way a straightforward procedure. Schrödinger called proteins "aperiodic crystals," to express the fact that proteins are structurally ordered, yet lack translational symmetry (1). However, things are not as simple as that. It is true that there is structural order as is, for example, documented by highly resolved x-ray scattering patterns of a growing manifold of proteins (2). Yet, proteins are far from being true crystals, mainly because their structural order pertains only on mesoscopic scales. Microscopically, proteins much more resemble glasses (3). On microscopic scales, disorder prevails, that is, some atoms of the protein do not have a well-defined equilibrium position but rather can reside in two or several metastable positions. This fact leads to a huge number of conformational states which are separated by conformational barriers. Another feature which shifts proteins close to glasses is that these barriers are distributed over a fairly large range (for a review see reference 4). There may be high barriers which keep the protein in a confined range of its phase space (5). However, evidence was found for the existence of rather small barriers on the order of a few tens of kilojoules per moles which the protein can surpass even at cryogenic temperatures (6–11). With increasing temperature the protein continuously changes its structure, such that more and more conformations become available. The range in phase space where the protein is allowed to move increases strongly with temperature. It seems that the fluctuations between a huge variety of microscopically different structures might be important for a proper functioning of the protein (7). How can the conformational substates and the related dynamics be probed? The nice thing is that several, easily measurable properties of a protein do obviously depend on conformational substates. Hence, they can be used to probe them. An example is the CO-stretch frequency in carbonmonoxy myoglobin (12). The reactive barrier between bound and deligated carbonmonoxy myoglobin depends on the conformation of the protein (12, 13). Literature data indicated a broad distribution of this barrier which was found perfectly Gaussian (14). The Gaussian distribution of this barrier is just an indication that there is no correlation between the region in the phase space where the protein moves around and this reactive barrier.

In this paper, we use a more direct technique to investigate conformational changes in proteins, namely the temperature cycling hole burning technique (15–17). This technique probes the relaxation processes of the protein in a most direct fashion by observing the spectral diffusion broadening of a burnt-in photochemical hole. In this paper we use the term "hole" in the sense as it was defined in classical NMR and optical saturation

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spectroscopy (18; for a review see 19). Some authors have recently used this term under a much different meaning (20). The hole, burnt at the lowest accessible temperature of the experiment, serves as a marker for a special comformational state (of course, this is an idealized assumption). At liquid He temperature the burnt holes may be extremely narrow, e.g., a few hundred megahertz. Hence, they are extremely sensitive to small structural changes around the photoreactive dye molecule which is used as a probe. In a temperature cycling hole burning experiment the temperature is raised after hole burning to a value T which we call the excursion temperature. Because of the temperature increase, the protein starts moving around in phase space. When the temperature is cycled back it gets trapped again, but this time in a different conformational state as compared to the initial state where hole burning was performed. Because the microscopic strain fields and electric fields may differ a little bit in the new state, the absorption frequencies of the probe dyes will be shifted a little bit. As a consequence the hole will broaden. This phenomena is called spectral diffusion broadening (21). Because the hole can be extremely sharp, broadening phenomena on the order of a few hundred megahertz can be easily measured. The broadening is irreversible with temperature. The irreversibility indicates in a most direct way that the part of the phase space which equilibrates during the temperature rise, falls out of equilibrium again when the temperature is cycled back. Hence, this kind of experiment demonstrates clearly the nonergodicity of the systems investigated.

So far, temperature cycling hole burning has been used only once to investigate conformational relaxation of a protein (11). In that case the protein was the phycobilisome from the algae mastigocladus laminosus. This is a large protein assembly with a molecular weight on the order of 5,000 kD.

In the present paper, we apply the method to a rather small protein, namely to one of the isoenzymes of horseradish peroxidase: HRP/C (simply: HRP). HRP is a heme glycoprotein which catalyzes the oxidation of a large variety of compounds in plant roots. Its molecular weight is 34 kD. To perform frequency selective photoreactions, the heme in the native enzyme was substituted by mesoporphyrin IX (MP). It was shown earlier that the enzyme maintains its substrate binding ability after the MP substitution (22). Laser excited fluorescence spectroscopy at low temperature performed on MP-HRP resulted in well resolved spectra indicating that site selection spectroscopic techniques can be applied to this protein (23). It is well known that free base porphyrins undergo phototautomeric transformations by switching the positions of the two inner protons. By using the fluorescence site selection technique, different $0 \leftarrow 0$ bands were identified as characterizing different tautomeric forms, and the inhomogeneous distribution of the $0 \leftarrow 0$ transition energies caused by conformational substates of the protein was determined for the individual tautomeric components (24, 25). The width of the distribution was found to be rather narrow, only 60 cm^{-1}. (Note that a width of ≈ 200 cm^{-1} was estimated for chlorophyll substituted myoglobin in reference 26.) In this paper we exploit the phototautomerization reaction in MP for a series of hole burning experiments with the general goal to learn something about conformational relaxation processes in this small protein.

MATERIALS AND METHODS

Materials

HRP/C was isolated from horseradish, purified, and recombined with MP by Paul (as described in references 27, 23), and kindly donated for our purposes. In this method, the pure protein fraction was split into heme and apoprotein by the two-butanone technique, and purified MP was combined with the apoprotein in an excess of 25% at pH 8, 4°C. After recombination, chromatography was applied to remove denatured protein molecules and excess of MP. The pure MP-HRP fraction was stored at 200 K. Samples were prepared in a concentration of 20 μM, in 20 mM ammonium acetate buffer, pH 5. For measurements, 200-μl samples were used containing 50% glycerol to assure transparency.

Spectroscopic techniques

MP in the HRP-apoprotein acquires several clearly distinct sites, some of which can only be populated photochemically or by thermal treatment of the sample (see below). Hole burning in MP-HRP was performed in three different origins, at 6,130, 6,222, and 6,250 Å, in the vicinity of the respective maxima. There is experimental evidence (to be published in detail separately) that the studied sites are representatives of different tautomeric forms of MP. The 6,130-Å site is within the distribution of the most intensely populated MP configuration under normal conditions at room temperature. The 6,222-Å site belongs to a distribution, which is populated to a much lesser extent. In our experiment, these species were photochemically enhanced before hole burning. The 6,250-Å site is not populated at room temperature, thus, it cannot be observed after a normal freezing procedure. It only appears after combined photochemical (24) and subsequent thermal procedure.

The burning laser was a ring dye laser system pumped by an Ar^{++}-laser (models Innova 90 and 699–21; Coherent Radiation, Palo Alto, CA). Its bandwidth was <3 MHz. It was used for burning holes as well as scanning the narrow holes. The maximum scan width was 30 GHz. The holes were detected in the fluorescence excitation spectrum. Because the photosensitivity of the system is relatively high, broadband photochemistry (a few wavenumbers) could easily be achieved with a Xe-arc lamp through a 1 m-monochromator. This method was used for generating new tautomeric species originally not distinguishable in the spectrum (for details of the photochemistry between the tautomeric sites at 6,222 and 6,250 Å see references 24, 25).

The sample was mounted in a He-flow cryostat (VSK 3-300; Leybold-Heraeus Vacuum Products Inc., Export, PA), whose temperature was varied between the burning temperature T_b and the

excursion temperature T. Burning was always carried out at 4 K. T, which is the parameter of the experiment, was increased in each cycle in steps of 1–2 K. The highest value was limited by the signal-to-noise ratio. Because the holes broaden and recover, there exists an upper value of the excursion temperature beyond which the holes get so shallow that the signal-to-noise ratio drops below a reasonable value. This maximum excursion temperature was in all cases ~30 K. The accuracy of the temperature control was within 0.1–0.5 K, dependent on the absolute temperature. In the experiments to be discussed, the measured quantity was the irreversible change of the hole width after a temperature cycle has been completed. It was determined by fitting a Lorentzian lineshape to the hole after each cycle. From the corresponding width, the initial width of the hole before the first cycle was simply subtracted. This simple procedure could be employed, because it turned out that a Lorentzian lineshape was in any case a reasonably good fit. Of course, simultaneously with the width of the hole, we measured its recovery (i.e., the change of its area). We think it important to stress that the change of the width is decoupled from the change of the hole area. The latter is indicative for the details of the phototransformation processes and the distribution of the reactive barriers (28) and, hence, is treated in a separate paper.

RESULTS

1. Broad band excitation spectrum of MP-HRP at 1.5 K

A survey of the excitation spectrum of MP-HRP measured by a spectral resolution of 0.1 nm at 1.5 K in the Q band region is shown in Fig. 1. Samples directly frozen from room temperature did not show a temperature dependent spectrum between 77 (24) and 1.5 K. Previous (24) and ongoing fluorescence site selection spectroscopic studies (discussed in detail in a separate paper) reveal the existence of several, distinct 0 ← 0 bands in the Q_X range of the spectrum (shown by arrows); some of them can be better seen enhanced in Figs. 2–5. Holes burnt into three bands at 6,130, 6,220, and 6,250 Å have a homogeneous linewidth <350 MHz (10^{-2} cm^{-1}) at 1.5 K. Because such a value is typical for pure electronic $S_1 \leftarrow S_0$ transitions, this supports the idea that these sites

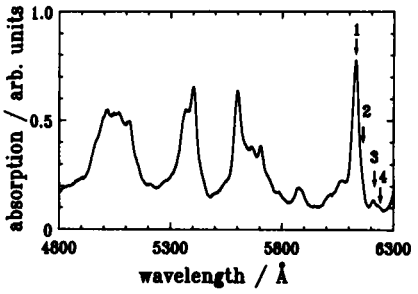

FIGURE 1 Fluorescence excitation spectrum (shown as absorbance) of MP-HRP at 1.5 K (or 4 K) in the Q band region measured by a resolution of 0.1 nm. Arrows indicate the position of tautomeric 0 ← 0 bands.

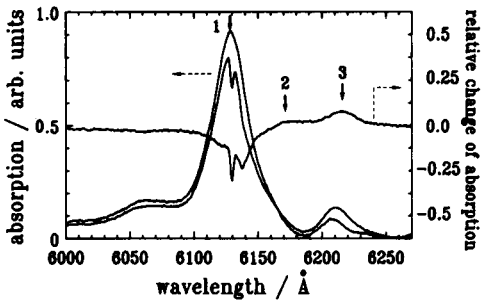

FIGURE 2 Enhancement of tautomeric bands 2 and 3 (*arrows*) through laser irradiation at 6,130 Å by 14 μW/cm², 5 min at 4 K. Spectra for the original condition and that after irradiation are shown.

represent different electronic origins of the S_1-state. Some of the 0 ← 0 bands are well resolved in Fig. 1, their estimated inhomogeneous linewidth at 1.5 K is 70 (band at 6,130 Å) – 60 (band at 6,210 Å, Fig. 4) cm^{-1}, very similar to the inhomogeneous width of an individual tautomeric form of MP in HRP, determined earlier by fluorescence site selection spectroscopy (24). This comparison shows that phonon wing transitions do not significantly contribute to the linewidth in this protein at low temperatures.

The special features in the spectrum shown in Fig. 1

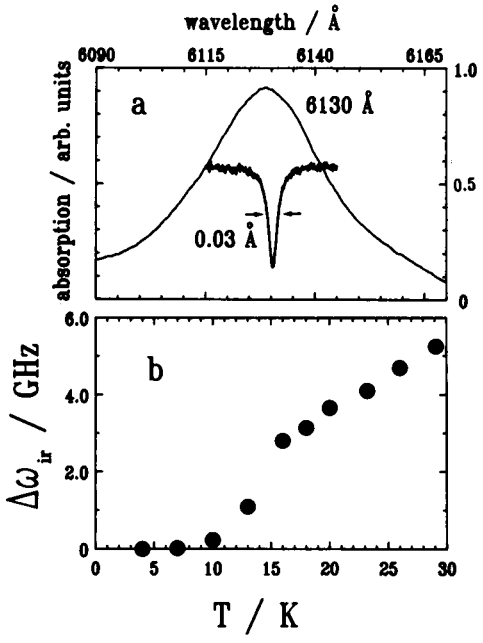

FIGURE 3 (*a*) Hole burnt in 0 ← 0 band 1 at 6,130 Å by 0.15 μW/cm², 6 min at 4 K. Wavelength scale for the hole is extended by 10^3, the depth is ~50%. (*b*) Change in the hole width ($\Delta\omega_{ir}$ "ir" stands for irreversible) plotted against the excursion temperature T in a temperature cycling experiment.

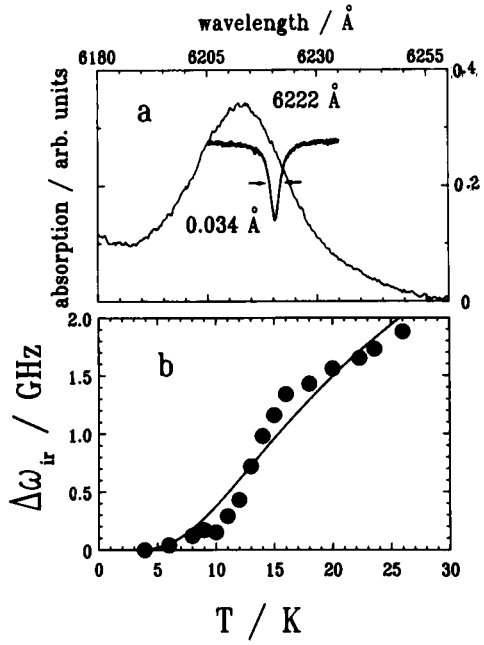

FIGURE 4 (*a*) Hole burnt in 0 ← 0 band 3 at 6,222 Å by 0.5 μW/cm², 7 min at 4 K. Wavelength scale for the hole is extended by 10^3, the depth is ~50%. (*b*) Change in the hole width $\Delta\omega_{ir}$ plotted against the excursion temperature *T*. The solid line represents a functional form for the change caused by thermal activation (see text).

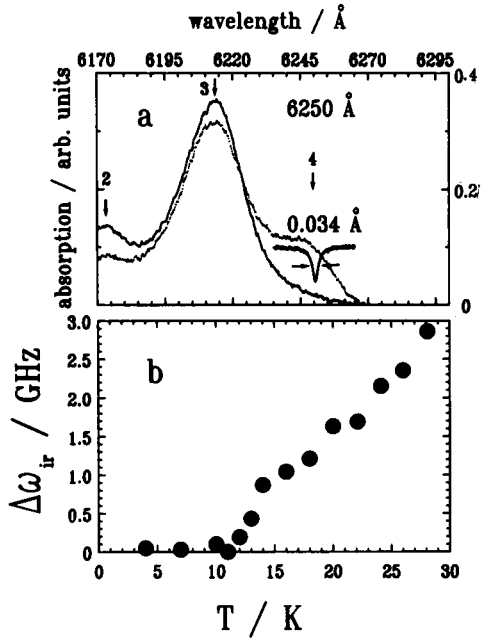

FIGURE 5 (*a*) Fluorescence excitation spectrum taken after photobleaching in band 1 at 4 K (spectrum characterized by bands 2 and 3), and the changed spectrum (characterized by a decrease in 2 and 3 band intensities and the appearance of band 4) after temperature cycling to 40 K, measured at 4 K. A hole burnt in band 4 at 6,250 Å is shown on an extended wavelength scale (by 10^3) with a depth of ~50%. (*b*) Change in the hole width $\Delta\omega_{ir}$ plotted against the excursion temperature *T*.

demonstrate that proteins are on the boundary between order and disorder. First, there are, like in crystals, discrete 0 ← 0 energies characterizing different tautomeric states of the chromophore, which point to a rather ordered structure of the heme pocket of the protein. Second, these discrete types of energies are, like in crystals, inhomogeneously broadened. The broadening (60 cm^{-1}) is significantly stronger than in crystals, but, on the other hand, is much smaller than a typical value of several hundred wavenumbers found in glasses (19). There is experimental evidence that HRP under different conditions (high pH or complexed with substrate) can show even narrower inhomogeneous distributions, namely, down to a width of 35 cm^{-1} (29).

2. Photochemical changes under irradiation

The conformers can be photochemically (and thermally) interconverted, but the photochemistry is in no case described by a simple two-state model. Fig. 2 shows an example of such a photochemical transformation. Irradiation into sites within the 6,130-Å spectral band by higher power irradiation (in this case: 14 μW/cm², 5 min, continuously scanning with the laser over 1 cm^{-1}) leads to the formation of a broad hole with characteristic phonon wing contributions and the enhancement of spectral bands 2 and 3 can be observed, as shown by the difference spectra in the figure. Using broad band irradiation with a bandpass of 30 cm^{-1}, and typically a power of 70 μW/cm² for 30 min through a monochromator, a 50% bleaching in band 1 ~6,130 Å can be achieved leading to the more pronounced enhancement of bands 2 and 3. Once the tautomer characterized by the spectral band at 6,210 Å was formed, a band at 6,250 Å (Fig. 5 *a*) also can be populated by simultaneous irradiation into the shorter wavelength products with high power (by three orders of magnitude) (24). Thus, photochemistry leads to the appearance of a series of chromophore configurations that can hardly be discerned in the original spectrum.

3. Characteristics of spectral holes in different tautomeric populations

The holes, which were investigated under temperature cycling conditions within three separate 0 ← 0 bands are shown in Figs. 3 *a*, 4 *a*, and 5 *a*. Also shown is the corresponding inhomogeneously broadened absorption

line. To convey an impression about the relative sharpness of the hole, which serves as a marker for the initial conformational state of the protein and as a probe for the phase space relaxation, note that the wavelength scale of the hole is enlarged by a factor of 10^3. For this kind of study, rather deep holes were initially burnt, hence, they were photochemically saturated to some degree. Their width is on the order of 0.07 cm^{-1}. Because in this experiment it was only the change of the hole width which was measured, its initial width is of minor importance. Before burning the hole at 6,222 Å, the population of the corresponding tautomer was photochemically enhanced as discussed above (Fig. 4 *a*).

The 6,250-Å band (Fig. 5 *a*) has special features. It appears not only by higher power irradiation, but also by thermal treatment (heating the sample up to 40 K) after the production of the 6,210 Å band. There is evidence that this band is an intermediate in the thermal relaxation of the photochemically generated higher transition energy species (Fig. 2, arrows *2* and *3*).

4. Line broadening during temperature cycling

The conformational relaxation processes of the protein show up in the thermally irreversible line broadening patterns of the hole markers as shown in Figs. 3 *b*, 4 *b*, and 5 *b*. As is immediately obvious from these figures, irreversible structural changes of the system do occur as the temperature is cycled between 4 and 30 K. The broadening pattern shows characteristic features: in all three sites, there is little broadening up to a temperature of ~ 10 K. Then, in Fig. 4, there is an evident steplike rise. Somewhat > 15 K, the broadening becomes weaker again. The steplike portion of temperature broadening is not equally significant for all tautomeric forms, though the same tendency is obvious. Besides these differences, the extent of the broadening effect is also different for the tautomers: 6 GHz is measured in the 6,130 Å site for a temperature cycle up to 30 K whereas 2 GHz is measured in the other sites. Presently we cannot exclude the possibility that apart from the 14-K step there are other steps at temperatures > 30 K. There are some indications which point to such a possibility.

DISCUSSION

1. Basic features of photoinduced proton tautomerism of porphyrinlike molecules in protein crevices

In this section we discuss the features of the overall spectrum and its related photochemical properties in more detail in order to characterize the special nature of this protein system with respect to its glasslike and crystallike behavior. This seems to be important for a qualitative understanding of its line broadening phenomena.

Though MP IX has substituents which destroy the symmetry of the parent porphyrin backbone, we will, for the following discussion, forget about this asymmetry. Such an approximation seems to be justified by the fact that the influence of the asymmetric substituents on the porphyrin absorption is dominated by the solute solvent interaction.

Suppose, we initiate a phototautomerization by a simultaneous rotation of the inner protons by $\pi/2$. Then, if the host is an amorphous glass which allows for all kinds of local configurations, we would never expect to measure a large site shift outside the inhomogeneous band of the photochemically produced product, because the product molecule is just the educt molecule rotated by $\pi/2$. Because all configurations are allowed, this special configuration must be already present in the inhomogeneous distribution. Hence, in an amorphous glass, a narrow bandwidth photochemistry of a porphyrinlike guest molecule resembles very much a photophysical hole burning system: the reaction is nothing else than a redistribution over the inhomogeneous band. An experimental evidence for this argument was recently published by Lee et al. (30) for the case of tetraphenylporphin in polystyrene. Of course, these arguments may soften a little bit in case the structural asymmetry and a more realistic phototransformation process (e.g., single and double proton jumps, out of plane configuration etc.) is taken into account.

Despite these complications, the situation in a protein crevice differs very much from an amorphous glass: to zeroth order, the chromophore frame has a fixed orientation with respect to the protein. Hence, in case the photoreaction is a double proton rotation, there are two discrete configurations, a situation very similar to crystals. Because the protein crevice does, for sure, not have the D_{4h} symmetry of the π-electron frame, those two configurations differ in energy. From the spectral features we conclude that this energy difference is on the order of 100 cm^{-1} (24).

A measure of microscopic order or disorder is provided by the inhomogeneous distribution function of $0 \leftarrow 0$ transition energies (31, 24), which in on the order of 60 cm^{-1}. This value shifts the protein-dye system away from a crystallike behavior into the neighborhood of glasses. Typical values in crystals are, for instance, a few wavenumbers. Typical values for glasses are, on the other hand, a few hundred wavenumbers.

The relatively large amount of inhomogeneous broadening (as compared to crystals) indicates, that the

relative geometry of the protein-dye system is by no means fixed. Instead, there is a random distribution of configurations around the discrete mean values. In case the photoreaction is described by a two-state model the sites must be photochemically and thermally interconvertable. However, we learn from the spectra (Figs. 2 and 5 *a*) that things are not as simple as that. A number of new species appear by irradiation into a single band (bands number 2–4 shown by arrows in Figs. 2 and 5 *a*).

Recently, a theoretical model was elaborated (32) for stable ground state geometries of free base porphin. By applying the theory of vibronic interaction to this system, it was shown that out of plane displacements for the pyrrole hydrogens are allowed and lead to four stable configurations of equal energy, each having the hydrogens at oppositely positioned nitrogens, one above and one below the porphyrin plane. These states may be mixed by intramolecular tunneling and local strains. There is in addition evidence for higher ground state energy tautomers having the hydrogens at neighboring nitrogen atoms (33, 34). Our studies on MP-HRP show four well distinguishable species separated in energy by ≈ 100 cm^{-1} interconverting under irradiation and heat treatment (peaks 1–4 in Figs. 2 and 5 *a*).

We conclude that the spectral features of the protein considered with the porphyrin probe enclosed in its pocket, reflect structural order as well as disorder. They are characteristic for a system between crystalline and glassy behavior. There is another point to be stressed: already the overall spectral features point to the fact that the protein pocket shields the dye molecule to a high degree from the solvent. So it seems that it is really the properties of the protein which are probed by the porphyrin. We will come back to this point below.

2. Basic features of spectral diffusion broadening of photochemical holes in glasses and proteins

After having discussed the overall spectral features of the protein-porphyrin system, and its spectral relation to microscopic order and disorder, we come to the main point of this paper, namely how detrapping and trapping processes in conformational phase space are reflected in spectral diffusion processes as measured by irreversible line broadening of burnt-in holes.

Two aspects are important in this context: first, how is the conformational phase space modeled, and second, how are the phase space relaxation processes reflected in the broadening of the hole.

As to the first question, we take the simplest approach. We assume that there are certain localized regions in the protein, which are structurally metastable. We assume, that the atoms which characterize such a region can reside in two distinct local minima. A transition from one minimum to the other would correspond to a simple conformational relaxation process. Hence, the phase space is modeled as an ensemble of such localized two-state regions which we consider as independent. This model is equivalent to the so called TLS-model which is well established in the science of glasses (35–37). A TLS (two-level system) would correspond to such a localized metastable region.

As to the second question, the situation is more difficult. Though the theories on spectral diffusion broadening in glasses are well established (38–41), even for the more complicated irreversible line broadening phenomena (21), a small protein is a different thing, mainly because of its finite size.

The basic physics which underlie the spectral diffusion models in glasses is the following: if a TLS, located somewhere in the glass, undergoes a structural transition, it creates a strain field (or, in case the atoms involved are charged, an electric field) which is felt by a probe dye molecule some distance apart. As a consequence, this dye will change its absorption frequency by a little bit. It is this change in optical properties which allows for an investigation of the structural transition. The fields generated by the structural transition are assumed to be of dipole character, both the strain fields as well as the electric fields. This is a rather long-range interaction which means that the contribution to the spectral shifts of the dye probe from distant TLS is appreciable, because their number is large. Because the overall concentration of TLS in a glass is rather low, the typical length scale of the interaction is large. It is assumed that on such a length scale, a glass is perfectly homogeneous. If all this fits together, it can be shown that a burnt-in hole will always be Lorentzian, irrespective of the changes it experiences by structural transitions. Its width has been shown to be proportional to the number of TLS which have changed their state during a temperature cycle (39). As to the temperature dependence of the spectral diffusion width under temperature cycling conditions, it has been shown that it is close to linear in T as long as the structural relaxation processes are tunneling processes, but increases by $T^{3/2}$ in cases, when activated structural processes are involved. It should be stressed that these temperature laws are a consequence of the smooth distributions of the parameters of the TLS, such as their energy asymmetry and their barrier height (21).

Three of the above assumptions, which work well for glasses, may break down in small proteins. First, because of their rather small dimensions, a small protein may not be perfectly homogeneous. Second, because of the mesoscopic size, the number of TLS is small (42) and, hence, there will be no appreciable influence of distant

TLS. Third, because of the small number of TLS, there are no smooth distributions of the TLS parameters like in glasses. Hence, the temperature dependent line broadening, because of structural transitions, may not be as smooth as in glasses but, may show steplike features.

Summarizing this section, we stress, that so far we have treated the protein molecules as being independent of the glycerol/buffer solvent in which they are dispersed. The dye probe is assumed to feel just the interaction with its protein bag, but is decoupled to a large extent from the solvent. Whether this is a reasonable view or not must be shown by the experiments.

3. Irreversible line broadening phenomena of spectral holes in MP-HRP

In this section we want to discuss the special features of the experimental results on MP-HRP in light of the above considerations.

First of all, the irreversible broadening of the holes, as displayed in Figs. 3 *b*, 4 *b*, and 5 *b* definitely shows that conformational relaxation does occur in the protein. The fact that there is no significant change in the hole width below 12 K supports the idea that the porphyrin is decoupled from the solvent to a large extent. The steplike increase of the widths ~14 K for all three holes may arise from the small size of the protein, and indicates that the structure of the protein may have changed slightly, and the porphyrin probe feels a different environment. That it did not appreciably change below 12 K is an indication that the protein does not have structural degrees of freedom that can be activated at these low temperatures. This contrasts markedly with glasses. We attribute this behavior to the finite dimensions of the protein. The structural change is not felt the same way by each of the tautomers. In this effect also, finite dimensions and inhomogeneities of protein structure may be involved. We also stress that it is not possible to perform reasonable fits to these data on the basis of the existing model for thermally irreversible spectral diffusion broadening. An example of such a fit, based on activated conformation relaxation processes is shown in Fig. 4 *b*. It is clear that the fit is not satisfactory.

Finally, a comment should be made on the lineshape of the holes under discussion. In our experiments, Lorentzian functions were used to fit the hole shapes. However, spectral diffusion theories show that Lorentzian lineshape is expected only in infinitely large and homogeneous systems. Our data seem to indicate that these conditions do not hold for HRP. We believe that the reason for the relatively good fit for Lorentzians in our case is that the holes were saturated to some degree. Saturation leads to lineshapes closer to Lorentzian, because it affects the wings of the holes broadening them artificially.

CONCLUSIONS

(*a*) Because there is such a pronounced difference between irreversible line broadening phenomena in glasses and small proteins, the porphyrin molecule in the protein crevice must be decoupled from the structural dynamics of the glassy solvent. A similar conclusion has been drawn from the characteristic features of the overall spectrum.

(*b*) Unlike a glass, a protein is not homogeneous as far as the TLS interaction is concerned. This is most obvious from the fact that the spectral diffusion broadening can be very different for the different sites. We stress that short range interactions (e.g. solvent cage effects) have, of course, a large influence on the site energy, not only in proteins (see Figs. 1 and 2) but also in glasses. However, the long range dipole-dipole interaction which governs spectral diffusion has a different length scale in which a glass, as stressed above, is perfectly homogeneous, whereas the protein is not. The steplike behavior seems to be due to a small number of TLS. It seems that the parameter distributions involved are not as smooth as in glasses, but may be discrete.

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