Interpretation of Multiple Q(0,0) Bands in the Absorption Spectrum of Mg-Mesoporphyrin Embedded in Horseradish Peroxidase

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ABSTRACT Mg-mesoporphyrin horseradish peroxidase (MgMP-HRP) and MgMP-HRP complexed with naphtohydroxamic acid (NHA) have been studied by fluorescence line narrowing (FLN) and pressure tuning spectral hole burning (SHB) techniques. In each sample, the low temperature absorption spectra show more than one transition in the origin range of the Q band. Comparisons with broad-band fluorescence spectra and FLN studies suggest that the multiple band feature originates from the presence of different configurations of the metal-porphyrin that are subject to Q_x-Q_y splitting within the protein cavity. This suggestion is supported by pressure tuning SHB studies. In the uncomplexed as well as in the NHA-complexed form of MgMP-HRP, irradiation in the Q band produces photoproduct bands, which has been attributed to a species with smaller Q_x-Q_y splitting. In an amorphous matrix, on the other hand, only one form of MgMP could be found, and no splitting could be observed. The binding of NHA does not significantly alter the bulk parameters of the protein matrix, but it reduces the structural variety in the configuration of MgMP to a single form with a more distorted structure and thus with an enlarged Q_x-Q_y splitting.

INTRODUCTION

It has been understood for a long time that the UV-VIS absorption spectroscopy of porphyrin-protein complexes is of great analytical value, and characteristic bands of the spectra have been used to follow, e.g., the oxidation state of the heme group in hemoproteins (Adar, 1978) or the details of photosynthetic reactions (Katz et al., 1978). The simple four-level model elaborated by Gouterman (1959) has formed the basis for understanding the origin of the spectral bands in the case of D_{4h} symmetry metal-porphyrins. In this model, the lowest energy visible α and the intense Soret band around 400 nm originate from electronic transitions from the highest filled molecular orbitals $a_{1u}(\pi)$ and $a_{2u}(\pi)$ to the lowest empty $e_g(\pi^*)$ orbital and lead through configuration-interaction to the lowest Q and higher B doubly degenerate excited states. The respective transitions from the ground state give rise to the so-called α and Soret bands. From the model, the energy separation of these bands and the relative oscillator strength can also be predicted. The so-called β band that appears at the high energy side of the α band has been understood as an envelope of vibronic transitions within the Q level (Perrin et al., 1969). When the spectra of metal-porphyrins embedded in proteins have been measured, however, characteristic deviations from the simple Q(α, β)-B assignment have been recognized and are being used as an analytical tool to differentiate between various ligated and oxidation states of the native heme group in myoglobin, hemoglobin, or various other enzymes (Adar, 1978). In some cases, the coordination of the metal-porphyrin to amino acids or different ligands within the heme pocket could be successfully interpreted as a totally symmetrical perturbation (electron donation to metal orbitals) within the frame of the four-orbital model (Gouterman, 1961; Shelnutt and Ortiz, 1985). However, from x-ray diffraction data it became obvious that interactions breaking the symmetry of an originally D_{4h} symmetry molecule must be present in native porphyrin-protein complexes, as in myoglobin and hemoglobin (Landner et al., 1977; Takano, 1977a,b). In *c*-type cytochromes, specific distortions conserved among mitochondria of various species have been found, and their relevance to the biological functioning has been suggested (Hobbs and Shelnutt, 1995). In the case of bacteriochlorophyll *a* molecules in *Prosthecochloris astuarii* antenna, the x-ray analysis showed that specific distortions of each macrocycle are introduced by the protein (Tronrud et al., 1986). The primary donors in the reaction center of *Rhodopseudomonas viridis* were also found to be conformationally distinct (Deisenhofer and Michel, 1989). The crystal structure of horseradish peroxidase (HRP), studied by us, is yet to be determined. However, results of electron paramagnetic resonance (Kobayashi et al., 1980) and Raman spectroscopy (Teraoka and Kitagawa, 1981) have indicated a pentacoordinated structure of the iron-heme. The heme in the peroxidase of the fungus *Arthromyces ramosus* was also found to be pentacoordinated and distorted from the planar structure by x-ray diffraction analysis (Kunishima et al., 1996). In fact, the inherent asymmetry of native metal-porphyrins (e.g., hemin) should already imply symmetry-breaking perturbations. Thus it should be the exemption rather than the rule that native metal-porphyrin protein complexes would show the simple Q(α,β)-B type absorption spectrum.

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In the literature, a number of examples can be found where multiple (0,0) bands in the Q(α) range of the absorption spectra of substituted and native hemoproteins are reported (Wagner and Kassner, 1975; Champion et al., 1976) and interpreted as the result of orientational disorder or of coordination effects (Friedman et al., 1977; Cowan and Gray, 1989; Kaposi et al., 1993). Some data suggest the presence of specific interactions: whereas a splitting has been reported for Zn-protoporphyrin-substituted sperm whale myoglobin (Cowan and Gray, 1989), in the spectrum of the same chromophore in horse myoglobin no such an effect has been found (Ahn et al., 1993). Although it was shown by quantum chemical calculations that the distortion of porphyrinoid molecules from the D_{4h} planar symmetry influence their redox and light absorption properties (Barkigia et al., 1988), spectroscopic evidence for symmetry breaking by proteins has not been presented.

In this work we have used low-temperature broad-band and high-resolution energy-selective spectroscopy to investigate the origin range of the Q band (i.e., Q(α)) in Mg-mesoporphyrin-substituted HRP and in the respective complex with naphtohydroxamic acid (NHA). At cryogenic temperatures, the increased resolution unravels the composite character of the Q band: a multiple band feature becomes evident. A comparison of absorption and fluorescence spectra suggested the presence of Q_x-Q_y splitting for both HRP and HRP/NHA with distinct energy separation. We have used the FLN technique to distinguish between bands of electronic and vibronic origin. From pressure tuning SHB experiments it also became evident that structural/coordinational states are hidden below the inhomogeneous envelope of the Q band. The identification of split bands was supported by the fact that no narrow holes could be burnt in the higher energy Q_y bands. Thus it seems to be clear that the degeneracy of the Q band is broken in both proteins, albeit to a different extent, and a splitting on the order of 100 cm^{-1} is present. The Q_x-Q_y splitting also changes when new configurations are generated photochemically. We interpret the structurally distinct species as the result of a change in the bonding network toward amino acids or other ligands (e.g., water) in the heme pocket. The significance of regulation by configurational/structural changes of the porphyrin ring is emphasized.

MATERIALS AND METHODS

Sample preparation

The enzyme horseradish peroxidase (HRP) was used as a protein matrix. Isoenzyme C of HRP has been isolated and purified from horseradish. The native hemin has been subtracted from the enzyme by acid methyl ethyl ketone (Teale, 1959), and the apoprotein has been reconstituted with Mg-mesoporphyrin (MgMP) from Porphyrin Products (Logan, UT) dissolved in ethanol. Samples of MgMP-HRP were prepared in a concentration of ~400 μM in 50 mM, pH 7 phosphate buffer containing 50% glycerol to ensure transparency. Modified protein samples were prepared by complex formation with the aromatic H-donor naphtohydroxamic acid (NHA). MgMP-HRP/NHA was prepared in the same buffer by adding aliquots of stock NHA dissolved in ethanol up to an excess of 5:1 molar ratio. A mixture of ethanol and glycerol with a volume ratio of 1:1 has been used as a glassy matrix for MgMP.

Spectroscopic techniques

Fluorescence emission was obtained by a FS900CD spectrofluorimeter (Edinburgh Analytical Instruments, Edinburgh, Scotland) with a resolution of ~10 cm^{-1}. Low-temperature absorption spectra were detected with a Jobin Yvon monochromator THR 1000 (Jobin-Yvon, Longjumeau, France) with a resolution of 2 cm^{-1}.

High-resolution emission spectra were obtained by using a Coherent 899-01 ring dye laser pumped by a cw Coherent Innova 300 argon ion laser. The power of the laser beam was attenuated to 1–2 mW. The emission spectrum was measured at 90° from the excitation by using a THR 1000M monochromator. The resolution of the spectrophotometer was 2–3 cm^{-1}. The sample was cooled to 10 K by a closed-cycle He refrigerator.

Spectral hole burning has been performed at 1.5 K in a He bath cryostat with a single frequency ring dye laser system. The spectral width of the laser was on the order of a MHz. Burning power and burning times were ~100 μW and 30 s, respectively. The holes were detected in transmission. During the pressure tuning experiment, the samples were kept at 1.5 K. Pressure was transmitted and regulated via He gas with an accuracy of 10^{-3} MPa and varied up to 1.1 MPa. Samples were sealed in small plastic bags to ensure isotropic pressure. The pressure cell was immersed in liquid helium.

Determination of the inhomogeneous distribution function by FLN spectroscopy

In the fluorescence line narrowing technique, a narrow-bandwidth tunable laser source is used to illuminate the sample at cryogenic temperatures, and molecules with vibronic transitions equal to the photon energy of the laser light are selectively excited. Emission occurs after vibrational relaxation. It is supposed that the chromophores are frozen in the distribution of protein conformations that manifests itself in the inhomogeneous broadening of (0,0) transition energies. It is also supposed that this inhomogeneous distribution of conformations does not influence the vibrational motions of the chromophore (Personov, 1983). These characteristics of the FLN spectra make possible the determination of the inhomogeneous distribution function (IDF), that is, the determination of the true (0,0) band deprived of phonon and vibronic contributions (Fuentschilling and Zschokke-Graenacher, 1982; Fidy et al., 1989; Kaposi et al., 1993). In this experiment, a series of emission spectra with shifted excitation frequency is measured, and resolved (0,0) lines originating from the same vibronic zero phonon excitation are evaluated. While they are followed through the whole (0,0) emission region, their intensity will be determined by the inhomogeneous distribution function. A correction is applied for nonresonant excitations via phonon sidebands that produce an unresolved background emission present in the spectra.

Spectral holes under pressure

Changes in the hydrostatic pressure influence the parameters of spectral lines. In this article, we focus on the pressure-induced line shift of spectral holes. The experiments were all carried out in the low pressure range, where proteins show perfectly reversible behavior (Zollfrank et al., 1991). The interpretation of the results has been based on the theory elaborated by Laird and Skinner for amorphous systems (Laird and Skinner, 1989). It has been shown experimentally that this model is applicable to proteins (Zollfrank et al., 1991) under the following conditions: 1) the protein is assumed to be a homogeneous isotropic matrix for the chromophore; 2) the atomic groups interact independently with the chromophore; 3) the chromophore-solvent interaction is described by the attractive part of the Lennard-Jones potential ($\sim R^{-6}$). This approximation also involves the concept that the pressure-induced shift of the hole is directly proportional to the solvent

shift caused by the protein. Within these approximations, the pressure shift of a hole is given by

$$s = 2\kappa\nu_s\Delta p \quad (1)$$

where κ is the isothermal bulk compressibility of the host protein, and Δp is the pressure change. ν_s is the solvent shift:

$$\nu_s = \nu_b - \nu_{vac} \quad (2)$$

where ν_b is the burning frequency and ν_{vac} is the vacuum absorption frequency of the chromophore, that is, the absorption frequency of the chromophore deprived of the protein. Because in this model the chromophore can only feel the compression of the lattice within the range of the relevant interaction forces, κ is defined as a local compressibility. The range of dispersion or higher order electrostatic interactions is very short, commensurate with the typical dimensions of the considered proteins. As a consequence, the optical technique measures directly the compressibility of the protein molecule.

RESULTS

Description of the samples

In the present studies, two kinds of protein samples have been investigated. The chromophore was Mg-mesoporphyrin in both cases. Horseradish peroxidase substituted with MgMP represented one kind of sample. A modified protein sample was prepared by adding naphtohydroxamic acid to MgMP-HRP, so that the saturation of primary binding sites has been achieved (Fidy et al., 1992). It has been established that a series of aromatic molecules are able to function as H-donors in the enzyme reaction of the peroxidase (Schonbaum, 1973; Schejter et al., 1976; Fidy et al., 1989). It is not known, however, how the binding of these molecules takes place, but during the multistep process of the oxidation reaction these molecules must be present at two succesive times within the heme pocket of the protein (Stillman et al., 1975). Based on the model elaborated for the binding of benzohydroxamic acid (BHA) (Sakurada et al., 1986) and previous studies concerning the spectral changes upon binding of NHA (Fidy et al., 1991, 1992), we believe that like BHA, NHA also binds into the heme crevice, but does not directly interact with the π system of the porphyrin. The effect of binding is seen in the absorption (emission) spectra (Sakurada et al., 1986; Fidy et al., 1989, 1992) and thus can be followed. In the present studies, the complex formation with NHA has been used as a tool to perform a slight modification of the original protein structure on one side, and on the other side, the complex with NHA is a structure of biological significance that is interesting to elucidate.

Comparison of broad-band fluorescence and absorption spectra at low temperature

A detailed comparison of fluorescence emission and absorption spectra has been performed in the case of MgMP-HRP and its complexed form with NHA. The emission spectra were determined at 10 K by using a closed-cycle He refrigerator, and the absorption spectra were measured at 1.5 K in a He bath cryostat. The spectra are shown in Figs. 1 *a* and 2 *a* for the free enzyme and the complexed form, respectively. Fluorescence emission was excited in the Soret band, using the widest slit width (18 nm) to make sure that all

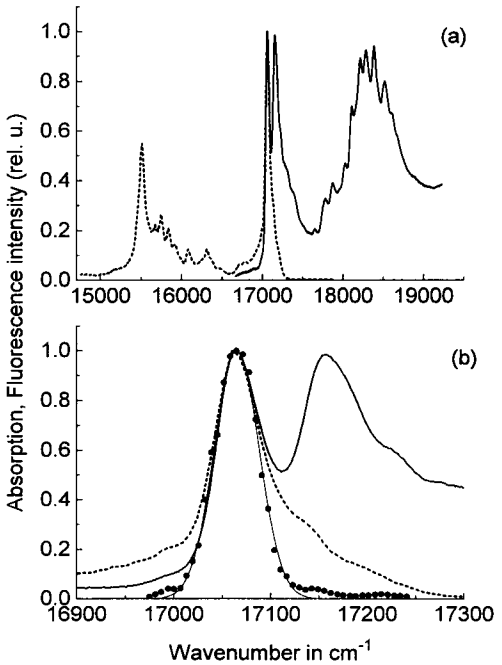

FIGURE 1 (*a*) Fluorescence emission excited in the Soret band (*dashed line*) and absorption (*full line*) spectra of MgMP-HRP. (*b*) The (0,0) range of the spectra, the data points of inhomogeneous distribution function and one Gaussian (*thin line*) fitted to the main component.

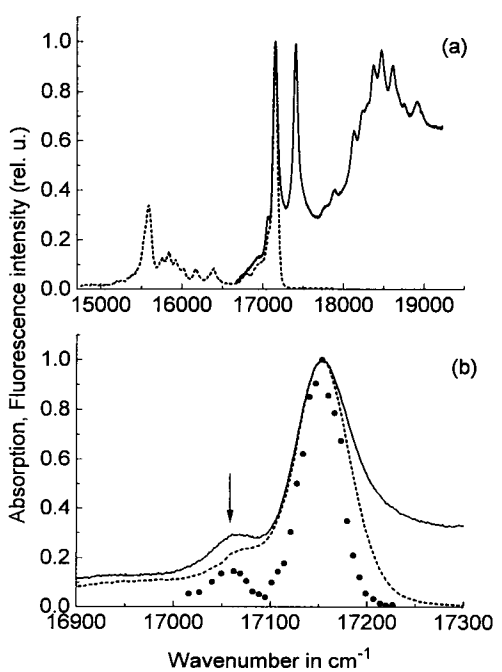

FIGURE 2 (*a*) Fluorescence emission (*dashed line*) and absorption (*full line*) spectra of MgMP-HRP/NHA. (*b*) The (0,0) range of the spectra and the data points of inhomogeneous distribution function. The marked band represents the contribution of uncomplexed enzyme molecules.

kinds of chromophores are equally excited and represented in the spectra. Significant changes due to substrate binding can be observed.

The absorption spectra in both cases contain two rather narrow, intense bands in the Q(0,0) range. The vibronic components of these spectra could be interpreted as a set of two vibronic series originating from two separate electronic components (after correcting for the increased scattering toward higher frequencies). The fluorescence emission spectra, however, contain only one intense band in the (0,0) range that overlaps with the lowest band in absorption spectra. In case of the free enzyme, a slight indication of shoulders on the high energy side of this intense band in the emission spectrum is also observable (Fig. 1 *a*). In the case of the complexed form, in both the emission and absorption spectra, an additional component (see the marked band in Fig. 2 *b*) can be identified at the low energy side. It is due to a small portion of uncomplexed free enzyme molecules that are still present in the sample.

Inhomogeneous distributions determined by FLN

In Figs. 1 *b* and 2 *b*, the (0,0) ranges of the spectra shown in Figs. 1 *a* and 2 *a* can be seen on a magnified scale. Overlaid on these spectra, results of FLN studies concerning the inhomogeneous distribution of the (0,0) transition energies are shown. In the FLN measurements, emission line intensities in the (0,0) range have been registered while tuning the exciting dye laser by steps of 6–7 cm^{-1} in the range of 17,900–18,400 cm^{-1}. In our case, the width of the distribution functions is 40 ± 5 cm^{-1}. It is known that the heme pocket in HRP represents a well-defined, rather rigid environmental configuration for the embedded prosthetic group (Brunet et al., 1990). Thus the IDF of an enclosed chromophore is significantly narrower than that found in, e.g., myoglobin (Boxer et al., 1987; Kaposi et al., 1993). The results are comparable to the low-temperature spectra. Our general conclusion is that although there seem to be two electronic origins in the absorption spectra, emission only from the lower energy level is found, both in the free and in the complexed forms of the enzyme. The IDF (Fig. 1 *b*) also shows clearly that besides the main component at 17,060 cm^{-1}, there are additional, only slightly populated emitting bands centered at 17,140 and 17,210 cm^{-1}, respectively, which are also observable in the broad-band emission spectra of HRP.

Photochemical changes

The origin of the multiple (0,0) bands has been investigated by irradiating into one of the bands and registering the changes in the absorption spectra. In Fig. 3 *a*, the difference spectrum before and after irradiation at 17,039–17,050 cm^{-1} in the lowest energy band of HRP is shown. In Fig. 3 *b*, the two spectra are overlaid for comparison. In the same way, results for HRP/NHA are shown in Fig. 4, *a* and *b*. In this latter experiment, irradiation has been performed at 17,140–17,151 cm^{-1}. In both cases, irradiation into the lower energy band leads to a bleaching of the higher band as well. Photoproduct appears between the two bands. In the case of HRP/NHA (Fig. 4), however, the photoproduct shows two well-resolved bands. It seems that in the free enzyme, the respective transitions are too close to be resolved. The photochemical transformation was much more effective in the case of HRP/NHA. Apart from this "broad-band" phototransformation, there is an additional one that occurs in a very narrow range of only a few GHz. It shows up in pronounced antiholes, as can be seen in Fig. 5. The nature of this transformation is not known, but we stress that it has been observed in other metal porphyrins as well (Köhler et al., 1996).

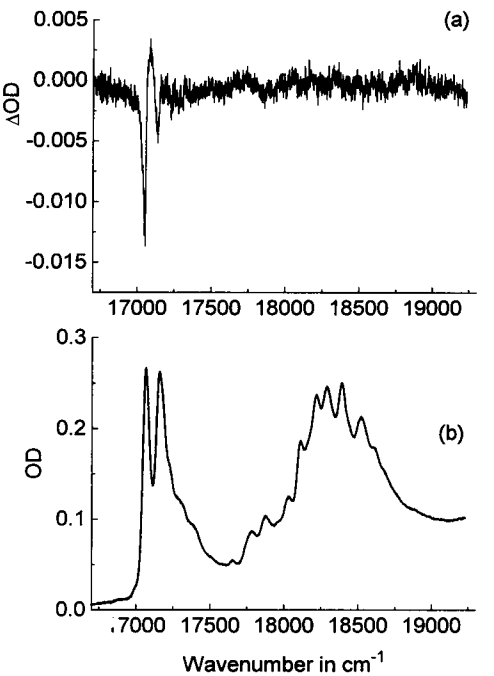

FIGURE 3 (*a*) The difference absorption spectra of MgMP-HRP before and after irradiation by laser scanning in a range of 17,039–17,050 cm^{-1} through 50 min with a power of 500 mW. (*b*) Wide-range absorption spectra. The solid and dashed lines (totally overlapping) represent, respectively, the spectra before and after irradiation.

Pressure tuning, spectral hole burning experiments

In previous works it has been shown that pressure tuning, hole burning techniques can be successfully applied to the study of proteins (Zollfrank et al., 1991, 1992; Gafert et al., 1993a,b, 1994; Friedrich et al., 1994). The experimental results lead to parameters such as the isothermal compressibility of the protein and the "vacuum" transition frequency of the chromophore or the solvent shift as described. In this study we intended to determine these parameters for the Mg-porphyrin chromophore and its surrounding protein.

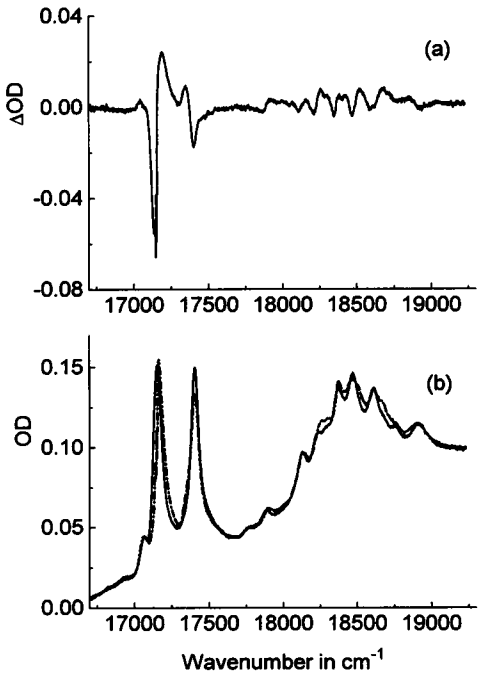

FIGURE 4 (*a*) The difference absorption spectra of MgMP-HRP/NHA before and after irradiation by laser scanning in a range of 17,140–17,151 cm^{-1} through 50 min with a power of 500 mW. (*b*) Wide-range absorption spectra. The solid and dashed lines represent, respectively, the spectra before and after irradiation.

In the case of the HRP sample, narrow spectral holes could be burnt at frequencies within both intense (0,0) absorption bands at 1.5 K; however, the burning efficiency was very low within the higher energy band. The hole spectrum had been registered at increased pressure values up to 1.1 MPa. An experimental series is shown in Fig. 5. The data show a perfectly linear dependence of the line shift within both bands (see inset in Fig. 5) in line with Eq. 1. The slope values of these straight lines at various burn frequencies within the absorption bands are plotted in Fig. 6 to yield κ from the slope of this plot and ν_{vac} as the frequency for

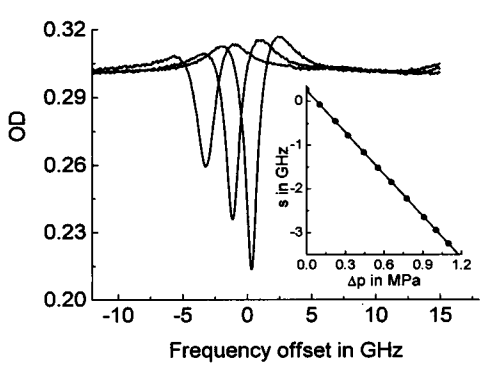

FIGURE 5 The hole burnt in the Q^I band of the absorption spectrum of MgMP-HRP at 17,055 cm^{-1} under varying pressure (at 0.0, 0.4, 1.1 MPa, respectively) at 1.5 K. (*Inset*) Shift of the center frequency of the hole with pressure.

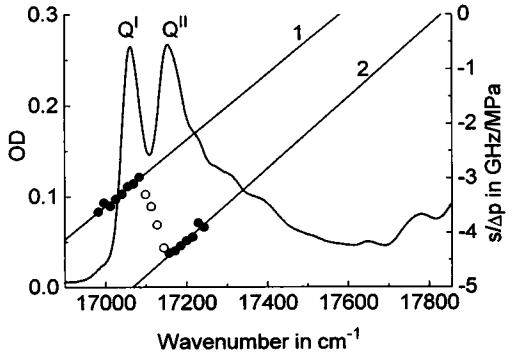

FIGURE 6 Frequency shift per unit pressure as a function of burning frequency within the inhomogeneous (0,0) band of MgMP-HRP. The straight lines (*1* and *2*) represent least-square fits to the data out of the overlapping region (*full circles*). The left and right scales represent the optical density and shift of the hole per unit pressure, respectively.

zero pressure effect. These data show two clearly distinguished components (1 and 2) in the pressure tuning experiments. These protein-chromophore complexes are characterized by very similar compressibility values (0.105 and 0.106 ± 0.005 GPa^{-1}, respectively) and, thus, by distinct vacuum frequencies. It is very interesting that the corresponding solvent shift frequencies are also definitely distinct. The conclusion is that in this spectral range there are two bands that correspond to two different structural configurations of MgMP in HRP.

The respective data for HRP/NHA in Fig. 7 are different from those of the free enzyme. In this case, narrow hole burning within the band around 17,400 cm^{-1} was not possible. The data within the main band around 17,150 cm^{-1} can be fitted well by a straight line. As mentioned earlier, the lowest energy small band arises most probably from HRP molecules not complexed with NHA. The compressibility value for the complex is slightly larger than that of the free HRP sample: 0.130 ± 0.005 GPa^{-1}. The solvent shift is similar (smaller by ~70 cm^{-1}) to that of the main component in the free enzyme.

Pressure tuning, hole burning experiments on Mg-MP in a glassy matrix

Results of the pressure tuning, hole burning studies on the chromophore dissolved in EtOH/glycerol are shown in Fig. 8. To evaluate κ, we took into account only the data related to the main band of the spectrum. The result of 0.100 ± 0.005 GPa^{-1} is very similar to that of MgMP-HRP and of other porphyrin systems—proteins or glasses (Zollfrank et al., 1991; Gafert et al., 1993a). It is evident that additional forms of the chromophore and matrix molecules are also present (shoulders in the absorption spectrum); however, their contribution is low and the pressure effect is not clearly resolvable, because of their spectral overlap. Spectral holes could be burnt over the main band and over the small additional bands with about the same effectiveness.

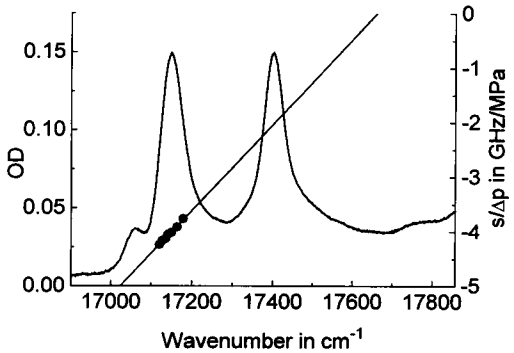

FIGURE 7 Frequency shift per unit pressure as a function of burning frequency within the inhomogeneous (0,0) band of MgMP-HRP/NHA. The straight line represents least-square fits to the data. The left and right scales represent the optical density and shift of the hole per unit pressure, respectively.

The vacuum frequency of the molecule falls into the inhomogeneously broadened absorption band; that is, the electronic transition of the Mg-MP is shifted only slightly by the matrix to the red (the solvent shift is $\sim 100\ cm^{-1}$) as compared to the case of the proteins, where significant red shift is observed. These results are somewhat contradictory to those reported by Lasagna et al. (1996) when the polarity of the heme crevice in HRP and that of EtOH as a matrix are compared. In those studies, however, the polarity was tested by fluorescent probes structurally different from MgMP.

DISCUSSION

Identification of (0,0) bands: Q_x-Q_y splitting

There is more than one piece of evidence that suggests the presence of a Q_x-Q_y splitting in the (0,0) range of the protein samples. Strong support comes from the photochemical studies that show the burning of the upper energy band through irradiation into the lower one (Figs. 3 and 4). The generation of a double product band by irradiation in the case of HRP/NHA shows that after phototransformation,

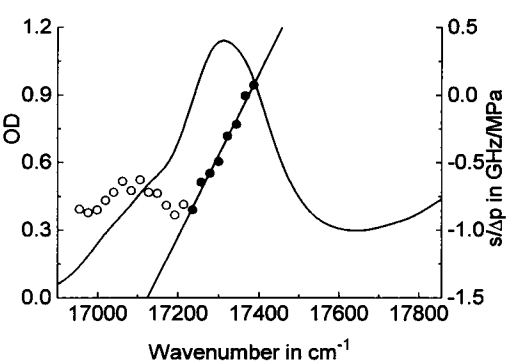

FIGURE 8 Frequency shift per unit pressure as a function of burning frequency within the inhomogeneous (0,0) band of MgMP-ethanol/glycerol.

the Q band may still be split, however, with altered energy separation. In the case of the uncomplexed protein, the respective splitting in the photoproduct state seems to be very small and unresolved (Fig. 3). The position of the spectral band of the photoproduct is in both cases identical with that of the unsplit transition band in the spectrum of the educt.

In the case of splitting in the Q band, it is expected that the upper level will be depopulated after excitation, mostly by internal conversion to the Q_x level, and emission will only be observed from this latter one. This is verified by the comparison of the low-temperature absorption and emission spectra, as shown in Figs. 1 and 2. Another consequence of the split nature of the two major (0,0) components in the absorption spectra of MgMP-HRP and of MgMP-HRP/NHA is that because of the fast relaxation from the Q_y level, the excited-state lifetime of this excitation would be very small. Thus spectral holes would be very broad and most probably would not be seen in the scanning range (30 GHz) of our experiments. This expectation agrees well with our finding that in the HRP/NHA sample, holes could not be burned within the higher energy (0,0) band, and in the case of HRP, the hole burning effectiveness was rather low in the upper band. (We argue—as we point out later—that the reason for hole burning in this case is that there is a hidden band of a conformational state with low intensity beneath the Q^{II} band, in which narrow hole burning is possible.)

The conclusion from Q_x-Q_y splitting is that while in a glassy matrix, only one Q band is observable for MgMP, interactions with the protein lead to symmetry breaking, and the degeneracy of the Q band is resolved. Literature data demonstrate that porphyrins intrinsically of D_{4h} symmetry when incorporated into proteins adapt a distorted structure. The significance of covalent bonds with the protein has been emphasized in the case of cytochromes *c* (Hobbs and Shelnutt, 1995), but nonplanar five-coordinate structure had been detected by x-ray crystallography, also in the case of a peroxidase with a heme noncovalently bound in the heme crevice. The heme distortion varied with pH and when ligands were bound to the heme (Fukuyama et al., 1995; Kunishima et al., 1994, 1996). Based on symmetry considerations, it can be expected that the out-of-plane distortions B_{1u} (ruffling) and B_{2u} (saddling) would be enough to break the degeneracy of the Q band. Thus we suppose that the observed Q_x-Q_y splitting is due to a nonplanar structure of MgMP in HRP. The energy separation is significantly different under different conformational states of the protein: in HRP it is 95 cm^{-1}, whereas in HRP/NHA it is 250 cm^{-1}. For the respective photoproducts it vanishes or is decreased to 150 cm^{-1}. These effects can be interpreted as consequences of the out-of-plane deformation of the porphyrin chromophore caused by noncovalent bonding to the protein under various conditions. There are indications of the presence of a network of hydrogen bonds around the heme in HRP (Smulevich et al., 1994).

Structural variety of MgMP stabilized by the protein

In the low-temperature fluorescence spectra (Fig. 1 *b*), it is seen that the lowest energy emitting Q band of the HRP sample contains more than one component. The contribution of additional components seen at the high-energy side of the emitting Q band (best shown by FLN) is small, but in the pressure tuning, hole burning experiments they become significant. In Fig. 6 it is clearly demonstrated by the experimental data that besides a population of MgMP-HRP molecules characterized by the intense lowest energy Q band, another population with the same compressibility but with a different vacuum frequency is also present (*curve 2* in the figure). The apparent hole burning efficiency for this second component was low, in accordance with the concept that the population where hole burning was possible is most probably identical to one of the small components in the IDF seen in Fig. 1 *b*. All of these observations and those discussed in the previous paragraph lead to the conclusion that in the range of the intense Q_y band, another Q band of a different MgMP-HRP population is also present. Thus Q^{II} in Fig. 6 is an envelope of this small band and Q_y. From the data in Fig. 1, one could even speculate that there might be more than one additional (0,0) band that could not be resolved in the hole burning experiment. The two solvent shift values estimated from the respective vacuum frequencies and the mean transition frequencies of the degenerate (unsplit) levels are 450 and 640 cm^{-1} for components 1 and 2, which differ significantly. This means that the additional population arises from differences in the local structure of MgMP as complexed with HRP. By comparing the absorption spectra of MgMP-HRP and MgMP/glass, we can exclude the possibility that the minority structural component arises from MgMP molecules attached externally to the protein. Thus the second component involves molecules with MgMP incorporated in the heme crevice, and it seems that the structural changes are not so severe as to change the bulk compressibility significantly. The conclusion is that there may be multiple ways for an embedded prosthetic group to become bound/coordinated to the protein, and these different forms are of quite definite character within a certain protein.

Effect of binding NHA

The experimental results concerning the effect of binding NHA are the following: the most significant change is seen in the Q_x-Q_y separation, and the Q band (average of the transition energies of Q_x and Q_y) is also shifted by ~200 cm^{-1} toward higher energies. The solvent shift is only slightly smaller than that of the main component in HRP without NHA. The compressibility is only slightly increased. Only one single structural component of MgMP coordinated to the protein crevice is observable when NHA is bound.

The general conclusion is that NHA introduces a "local" structural effect on the complex of the Mg porphyrin chromophore and the protein. The solvent shift and compressibility as bulk parameters of the protein are not affected very much, whereas the Q band splitting is significantly increased. This can be interpreted as the consequence of an increased out-of-plane distortion of the porphyrin. This explanation is in agreement with the structural model based on NMR data concerning the binding of BHA to HRP, which indicate that BHA affects mostly the peripheral 8-methyl group of the porphyrin (Sakurada et al., 1986). It is reasonable that such a steric costraint would lead to an increased out-of-plane deformation by affecting the position of the IV pyrrol ring. An increased distortion may also lead to an observable shift in the transition energy of the Q band, as was shown by Barkigia et al. (1988), which explains why we also detected a spectral shift along with the increased splitting effect when NHA was bound. The specificity of complex formation with NHA is underlined by the observation that in the presence of the substrate, the porphyrin structure is unambiguously determined.

The results concerning the effect of NHA binding on the structure of HRP outline different structural consequences when a metal-porphyrin or a free-base porphyrin substitution is studied. In our previous studies, a significant increase in the compressibility has been found when NHA is bound into MP-HRP (Fidy et al., 1992). The comparison suggests that the structural features of the complex of a free base- and metal-porphyrin with the surrounding protein may be significantly different. We believe that MgMP as a substituent is a better model of the native enzyme.

Changes upon irradiation

The results shown in Figs. 4 and 5 indicated that an excited-state transformation of the chromophore can be detected in both kinds of protein samples. If one also considers the feature of hole burning shown in Fig. 6, the conclusion is that the nature of this transformation is composite—it is both photochemical and photophysical in nature. We interpret the photochemical changes, i.e., we deal with the product bands created at an intermediate position between the split components of the educt. We believe that the photochemical change is similar in HRP and HRP/NHA, the new bands are characterized by a transition energy identical to that of the educt, but the splitting is significantly reduced. In the literature it was shown by electron paramagnetic resonance spectroscopy (Regev et al., 1994) that in the triplet excited state of octaethyl tetraphenyl porphyrin embedded in a liquid crystalline matrix, structural transition can be detected. Excited-state rearrangements of the pyrrol hydrogens in porphin have also been suggested (Völker and van der Waals, 1976; Völker and Macfarlane, 1980). In our experiments, the spectral changes upon irradiation were less pronounced and opposite those seen in the case of NHA binding. Thus the explanation can be an excited-state trans-

formation of the porphyrin into a slightly different, less distorted structural species. However, spectroscopic and theoretical studies by Platenkamp (1982a,b) showed that changes in both the energy of the Q band and the magnitude of the Q_x-Q_y splitting upon irradiation in the case of Mg-porphin embedded in *n*-octane can be interpreted as the consequence of an altered configuration of Mg-porphin and coordinated EtOH in the excited state. As we add MgMP to the apoprotein from a stock solution prepared in EtOH, we cannot exclude this possibility either; however, the similarity between the effect of NHA and irradiation suggests that an interpretation based on structural transitions between distorted configurations is more probable.

CONCLUSIONS

In this work we performed an analysis on the long-wavelength absorption band of a Mg-porphyrin chromophore embedded in a glassy matrix and in proteins. The comparison of low-temperature broad-band absorption and emission spectra, energy selective spectroscopic studies by FLN, and pressure tuning SHB revealed the nature of the multiple (0,0) bands seen in the spectra of the protein samples. The specific nature of the complex formation between the Mg-porphyrin and the protein is also demonstrated: conformation-sensitive symmetry breaking is observed (which manifested itself in the magnitude of the Q_x-Q_y splitting), and specific porphyrin configurations selected and well defined by coordination to the matrix have been found. We believe that this structural variety is most probably also present in hemoproteins built up by metabolic processes. The results on the HRP-substrate complex suggest that the functioning form of these molecules is strictly regulated by structural parameters that affect their electronic states.

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