Energy Selection Is Not Correlated in the Q_x and Q_y Bands of a Mg-Porphyrin Embedded in a Protein

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ABSTRACT The Q_x-Q_y splitting observed in the fluorescence excitation spectra of Mg-mesoporphyrin-IX substituted horseradish peroxidase (MgMP-HRP) and of its complex with naphthohydroxamic acid (NHA) was studied by spectral hole burning techniques. The width of a hole directly burnt in the Q_y band and that of a satellite hole indirectly produced in Q_y as a result of hole burning in Q_x was compared. We also studied the dependence of the satellite hole in the Q_y band on the burning frequency used in the Q_x band. Both the directly and indirectly burnt holes were very broad in the (higher energy) Q_y band. The width of the satellite hole in the Q_y band was equal to the entire width of the inhomogeneously broadened band, independently from the position of hole burning in Q_x. This is indicative of a clear lack of correlation between the electronic transition energies of the Q_x and Q_y bands. A photoproduct was produced by laser irradiation of the MgMP-HRP/NHA complex and was identified as a species with lowered Q-splitting. Conversion of the photoproduct could be achieved by thermal activation measured in temperature-cycling experiments, with a characteristic temperature of 25 K. We attribute the phototransformation to a conformational change of MgMP.

INTRODUCTION

It is known that due to the very short excited-state lifetime of the heme, the homogeneous spectral lines are very broad and the fluorescence emission is weak, and thus this chromophore cannot be studied by high-resolution optical methods. Some first successful spectral hole burning in a protein matrix was observed in c-phycocyanin and in the Zn-pyrochlorophyllide a−myoglobin complex (Friedrich et al., 1981; Boxer et al., 1987). In our previous studies (Friedrich et al., 1994; Balog et al., 1997; Fidy et al., 1998), we were, however, able to use these methods by substituting mesoporphyrin-IX (MP) and Mg-mesoporphyrin-IX (MgMP) for the heme in horseradish peroxidase (HRP). K_d measurements for substrate binding to the substituted enzyme yield somewhat increased K_d values, but only by one order of magnitude, thus showing that the MgMP-HRP studies have relevance for the native form and that the Mg-derivative of HRP is indeed a good analog of HRP (Balog et al., 2000).

The absorption spectra of metalloporphyrins under D_{4h} symmetry are usually qualitatively interpreted by considering only four orbitals, the two highest occupied orbitals of a_{1u} and a_{2u} symmetry and the doubly degenerate lowest unoccupied orbital of e_g symmetry (Gouterman, 1978). This accounts for two doubly degenerate electronic transitions observed as the Q band in the visible and the Soret band in the near UV range. Symmetry lowering can be achieved by ligand binding to the metal (Canters and van der Waals, 1978; Jansen and Noort, 1976), by substituting two hydrogens for the metal (Volker and van der Waals, 1976; Volker, 1987), or by asymmetric interaction with nearby atomic groups, as observed in Shpol'skii crystals (Dicker et al., 1983; Canters et al., 1972, 1973; Shelby and MacFarlane, 1979; Platenkamp and Noort, 1982; Platenkamp, 1982) and in proteins (Jentzen et al., 1998; Kohler et al., 1997; Balog et al., 1997, 2000). Any of these processes may lift the degeneracy, resulting in the splitting of both the Q and the Soret band.

Fluorescence emission detected from the lowest-energy band (Q_x) but not from the higher one (Q_y) convincingly shows that the observed Q-bands are not two distinct (0,0) bands originating from two different structural conformations. If they had been, obviously, they would not be related through energy relaxation (Balog et al., 1997; Dicker et al., 1983; Canters and van der Waals, 1978; Platenkamp and Noort, 1982; Platenkamp, 1982; Manas et al., 1999, 2000). It is possible, however, for the components of the split Q pair to overlap with the (0,0) bands of other structural features also present in the sample (Balog et al., 1997; Shibata and Kushida, 1998). The fact that the fluorescence yield is very low for iron porphyrins makes the spectral identification of the bands in such cases questionable. For example, the broad absorption Q band of some species of native cytochrome c was shown to split into two narrow resolvable bands at low temperature, and the energy separation was sensitive to the species (Laberge et al., 1998). The separation of the two bands was correlated to a theoretical model proposed to account for Q-splitting in such protein systems (Manas et al., 1999, 2000), although the split nature of the compared bands was not experimentally verified. Previous work, including our own studies (Balog et al., 1997; Shibata and Kushida, 1998) indeed shows that significant experimental efforts are required to achieve clear and unambiguous Q-splitting assignments. We believe that

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the Q-splitting, when carefully identified, represents an informative spectral parameter that can be used to understand how the biochemical activity of a prosthetic group is regulated in proteins.

For MgMP-HRP, we have previously shown (Balog et al., 1997) that the two spectral bands observed in the lowest energy range of the fluorescence excitation spectrum originate from the splitting of the Q band by an energy of 90 cm^{-1}. The observed splitting increases to $\sim$260 cm^{-1} upon binding a substrate of the native enzyme, naphthohydroxamic acid (NHA) in the heme pocket. Based on the recently published x-ray structure of the native enzyme bound to a similar substrate (Henriksen et al., 1998) and on our own isothermal compressibility and Stark effect experimental evidence (Balog et al., 1997, 2000), we can reasonably suppose that the binding of the substrate has probably no significant effect on the overall conformation of the protein. The biochemical function of the enzyme is carried out by locally modifying the electric field in the heme pocket as shown by an enlarged Q-splitting and a red shift of the spectrum. Our results on this protein system are comparable with earlier data reported on Mg-porphine embedded in a Shpolskii matrix (Dicker et al., 1983). The authors identified four different sites (or conformations of the chromophore) in the crystal matrix, each with significantly different Q-splitting. Q-splitting was also recently observed and assigned in the spectra of Zn-protoporphyrin substituted myoglobin and Zn-cytochrome c (Shibata and Kushida, 1998).

In this paper, we present new experimental results on MgMP-HRP by using the method of spectral hole burning. In this technique we spectrally label a certain subpopulation of the chromophore so as to investigate the nature of the Q-splitting within the inhomogeneously broadened spectra. The present experimental technique makes it possible to determine the $Q_y \rightarrow Q_x$ relaxation time from the homogeneous spectral line width (Dicker et al., 1983; Sapozhnikov, 1987; Avarmaa and Rebane, 1985; Volker and MacFarlane, 1979; Dicker and Volker, 1982) on the picosecond time scale. Temperature cycling experiments in the 5–45 K range were also performed to investigate the reversible phototransformation occurring in the substrate complex of MgMP-HRP.

MATERIALS AND METHODS

Sample preparation

Isoenzyme C of HRP was isolated and purified from horseradish roots. The pure fraction was treated with 2-butanone (Teale, 1959), and the apoprotein was recombined with purified Mg-mesoporphyrin (MgMP) (Porphyrin Products, Logan, UT) dissolved in ethanol. Samples were prepared and stored in 50 mM phosphate buffer, pH 7. To ensure optical transparency, glycerol was added (50% v/v); thus the final concentration of MgMP-HRP was $\sim$20 μM.

Modified protein samples were prepared by complex formation with the aromatic H-donor NHA. MgMP-HRP/NHA was prepared in the same buffer by adding stock of NHA dissolved in ethanol up to a [NHA]/[HRP] molar ratio of 5:1.

Spectroscopic techniques

Fluorescence excitation spectra and spectral hole burning experiments were carried out by a spectrometer setup consisting of a Coherent CR-490 tunable linear dye laser (bandwidth $\approx$ 0.5 cm^{-1}) with Rhodamine 590 (Exciton Inc., Dayton, OH) pumped by an argon ion laser Innova 70–4 (Coherent Inc., Santa Clara, CA) as described earlier (Avarmaa and Rebane, 1985). The dye laser output power was stabilized over the whole region of dye lasing (568–625 nm) and power density at the sample was kept in the range of 0.1–10 mW/cm^2. To calibrate the excitation spectra, the optogalvanic effect of a neon spectral lamp was used. The fluorescence signal was recorded by an RCA 31034A photomultiplier in photon-counting mode and accumulated in a multichannel analyzer (Nokia LP4900B). Spectra were recorded by scanning the laser frequency over the inhomogeneous bands of interest and detecting fluorescence through a cutoff filter at wavelengths >625 nm.

Spectral holes were burnt in the bands with the tunable dye laser. Burning power densities were 5–200 mW/cm^2 and the burning times varied between 20 s and 20 min. The holes were subsequently probed by scanning the excitation spectrum with the same laser at $\sim$100-times reduced intensity.

All spectra were acquired at 5 K using a temperature-controlled He immersion cryostat. For the temperature-cycling experiments, the sample was warmed up to a given temperature at which it was maintained for 5 min; it was then cooled to 5 K and the spectrum was recorded.

RESULTS

The fluorescence excitation spectra of MgMP-HRP and of its complex with NHA acquired at 5 K are shown in Fig. 1. In both spectra, the two intense Q_x and Q_y transitions can be clearly distinguished as assigned in our previous study (Balog et al., 1997), in which we also showed that in the HRP sample, the MgMP chromophore adopts two configurations in the heme pocket. The majority of the molecules correspond to the lowest-energy Q_x band, observed at 17,055 cm^{-1} (Fig. 1 *a*). The lowest (0,0) band of the second, less populated structure, however, overlaps with the higher-energy Q_y band of the first configuration at 17,155 cm^{-1}. Thus, in the case of the uncomplexed MgMP-HRP species, the second band (labeled as Q_y) has double character. When the substrate NHA is bound in the molecule, only one characteristic structure of the porphyrin is possible, and thus the two lowest-energy bands observed at 17,150 and 17,400 cm^{-1} (Fig. 1 *b*) are of purely Q_x and Q_y character. It is clearly seen that the extent of Q-splitting is significantly increased upon substrate binding.

In uncomplexed MgMP-HRP, spectral hole burning was very inefficient. We could observe only a narrow (laser-limited width) and shallow hole at the maximal burning flux of 200 mW/cm^2, which starts to saturate the burning at burning times of some minutes. No resonant spectral holes were observed when burning into Q_y (except a narrow hole due to the accidental coincidence of the Q_x band of the second overlapping structural configuration).

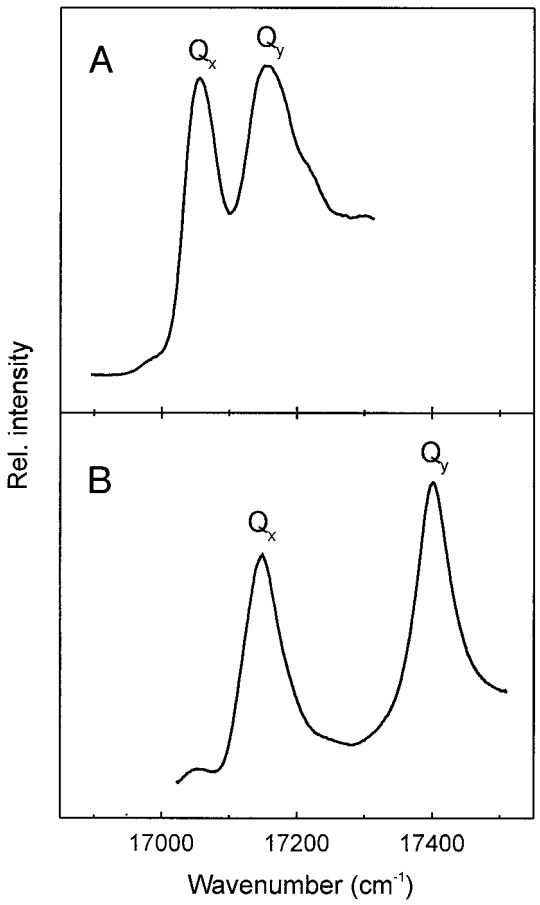

FIGURE 1 (*a*) Fluorescence excitation spectra of MgMP-HRP and (*b*) of its complex with NHA in the Q region at 5 K. (Spectra were recorded by scanning the laser frequency over the inhomogeneous bands of interest and detecting fluorescence through a cutoff filter at wavelengths >625 nm.)

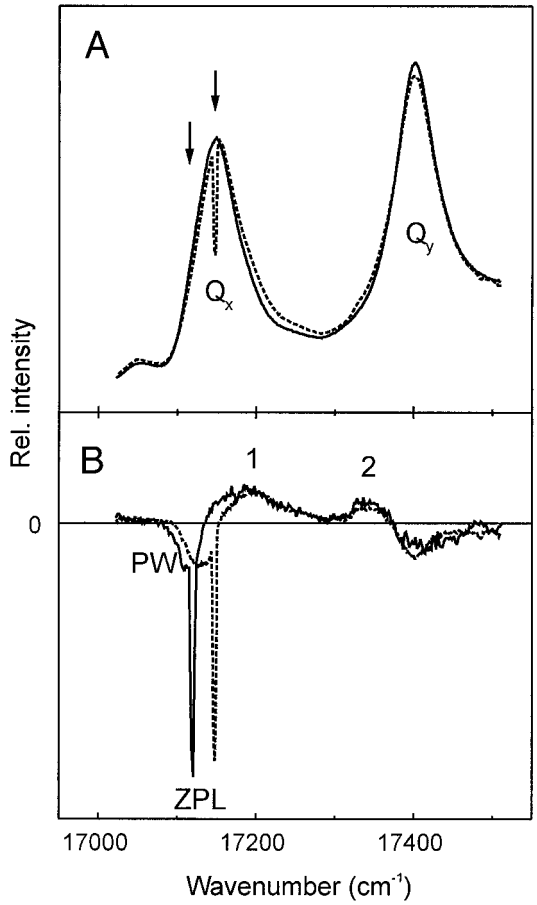

FIGURE 2 The hole burning effect in the Q_x band of the protein substrate complex (MgMP-HRP/NHA). (*a*) The spectrum before burning (——) and after burning at one frequency (- - -); arrows mark the burning frequencies. (*b*) The magnified difference spectra corresponding to the holes shown in *a* and taken after burning at two different frequencies (marked by *arrows* in *a*).

Fig. 2 *a* shows the effect of one hole burning experiment in the Q_x band of the substrate complex (MgMP-HRP/NHA) where the porphyrin was much more photoconvertible than in the protein without substrate. A laser-limited sharp zero phonon line (ZPL) and accompanying phonon wing (PW) is produced in the Q_x band as well as a broad decrease of the whole inhomogeneous band takes place in Q_y as the consequence of burning in Q_x. One can clearly see this effect in Fig. 2 *b*, where the magnified difference spectra are shown. The emerging bands are labeled as peaks 1 and 2 (at 17,190 and 17,340 cm^{-1}, respectively) and we assign them to a photoproduct with smaller Q-splitting (Balog et al., 1997; Platenkamp and Noort, 1982; Platenkamp, 1982). In Fig. 2 *b*, we also show the difference fluorescence excitation spectra after burning at another frequency in the Q_x band (see arrows in Fig. 2 *a*). It is clearly seen that the burning frequency within Q_x does not affect the burning in Q_y. The photoproduct also does not seem to change with the varying burning frequency.

Fig. 3 *a* shows a series of spectral holes at different burning doses. It is clearly seen that narrow holes cannot be produced in Q_y, where the spectral hole width is as broad as 13% of the whole inhomogeneous bandwidth. The line shapes at different burning doses are shown in Fig. 3 *b*. Because of the photoproduct, the left part of the holes (at smaller wave numbers) is rather distorted and the respective data points were excluded from the Lorentzian fitting. Extrapolation of the dose dependence of the hole width to zero yields a homogeneous line width of 3.5 cm^{-1}, which can be used to evaluate a Q_y-Q_x relaxation time of 1.5 ps with the well-known time-dependent perturbation theory relation.

Fig. 4 *a* shows the excitation spectra of the substrate complex (MgMP-HRP/NHA) recorded at 5 K after cycling to various temperatures. Before temperature cycling, a hole was burnt within a wavelength interval of 2.5 Å (~7.3 cm^{-1}), close to the maximum of the Q_x spectral band, by scanning the laser at 5 K. It is seen that the holes get refilled due to thermally activated energy barriers (activation enthalpies, *H*) crossing between the educt and a product state.

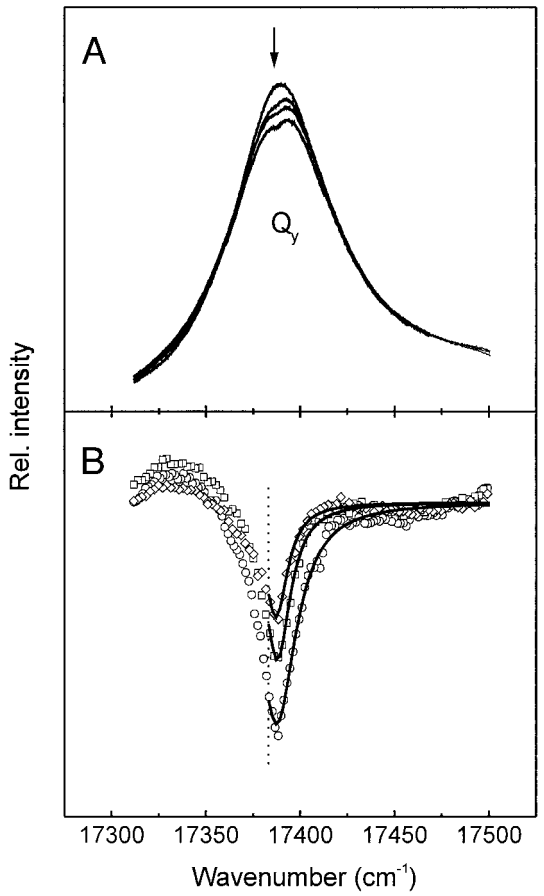

FIGURE 3 A series of hole burning experiments of the protein substrate complex (MgMP-HRP/NHA) in the Q_y band. (*a*) Successive spectra showing the effect of increasing burning time (at constant intensity). (*b*) The series of magnified difference spectra (holes) shown by open symbols, with the fitted Lorentzians shown by solid lines.

This product is characterized by the excitation spectrum observed as positive bands (antiholes) in the difference spectrum of Fig. 2 *b* (peaks 1 and 2). The hole area is proportional to the amount of molecules that do not cross the barriers at a given temperature T during the waiting time t. The distribution of energy barriers P_H is given by the following formula:

$$P_{\mathrm{H}} = -\frac{1}{A_0}\frac{dA_{\mathrm{T}}}{dT}\frac{1}{k\ln(R_0 t)}, \qquad (1)$$

where A_0 and A_{T} are the hole areas at the beginning and after the cycling to a temperature T, R_0 is the pre-exponential rate factor in the kinetics of the activated process, and k is the Boltzmann constant (Kohler et al., 1988; Fidy et al., 1992). After hole burning at 5 K, the sample was warmed up to a certain cycling temperature and kept there for 5 min; then it was cooled back to 5 K where the spectrum was recorded. The dependence of the hole area on cycling temperature is shown in Fig. 4 *b*. The corresponding activation energy distribution function is shown overlaid on these data as calculated from Eq. 1. The center of the distribution function is at 25 K with a FWHM of 13 K. This corresponds to a rather low energy barrier between the two states (Herenyi et al., 1995).

DISCUSSION

Phototransformation of Mg-mesoporphyrin-IX in HRP

The mechanism of nonphotochemical hole burning in solids presupposes the presence of a given free volume in the surroundings of the chromophore so as to stabilize a new configuration for the system. Usually, the nonphotochemical transformation is observed in disordered glassy systems. In hemoproteins, the effect of disorder translates into an ability of the system for hole burning. The experiments reported in this work clearly show that the hole-burning efficiency is lower in the substrate-free enzyme when compared with the sample with substrate. That is, the uncomplexed system seems to be more rigid than the sample with NHA. In addition, the substrate-free MgMP-HRP is characterized by stronger electron-phonon interaction, which strongly decreases the efficiency of the zero phonon hole burning.

In the MgMP-HRP/NHA complex, burning into Q_x produces two new spectral bands between the original Q_x-Q_y pair. The slight or absent spectral shift of the overall position of the Q band (average of band positions) suggests that the structural change involved in this transformation is very slight; however, the Q-splitting is sensitive to the change. Some comparable results were reported for the Mg-porphine system as well (Dicker et al., 1983). Irradiation into Q_x in one of the crystal sites (with larger Q-splitting value) led to a transformation into a species with lowered but still pronounced Q-splitting. The authors mention that they could not achieve nonphotochemical transformation in the other crystal sites, as we observed for the uncomplexed MgMP-HRP system.

The blue shift of the Q_x band and the decreased Q-splitting of the photoproduct of the MgMP-HRP/NHA complex are consistent with a mechanism of nonphotochemical hole burning based on an outside-in hierarchy of constrained configurational tunneling events (Shu and Small, 1990). According to this model, the photoproduct is characterized by an increased free volume.

Our temperature-cycling experiments show that the phototransformation is thermally reversible. The very low energy barrier is comparable with that found for some pyrrole tautomer forms of free base porphyrins that were photochemically generated and trapped at low temperature in proteins (Fidy et al., 1992; Herenyi et al., 1995).

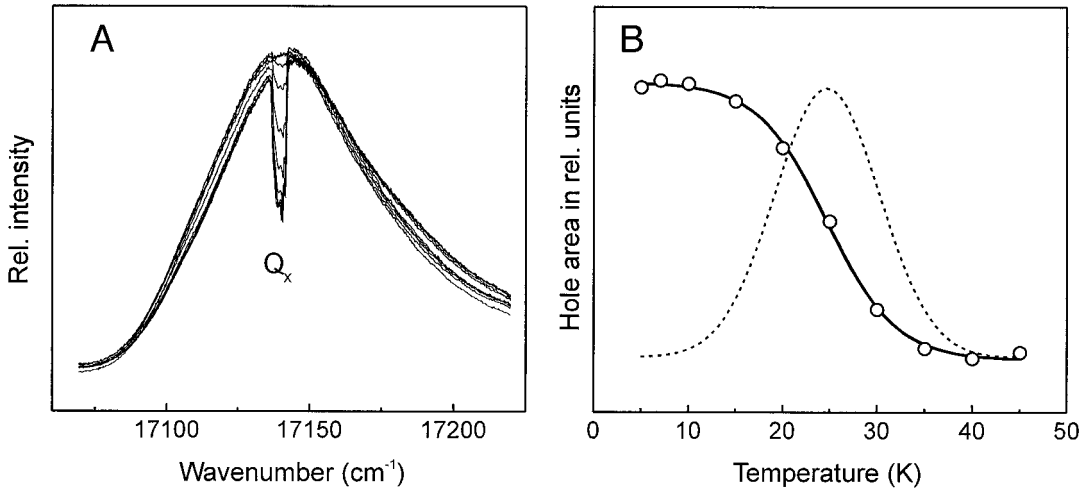

FIGURE 4 (*a*) The temperature-cycling experiment of the protein substrate complex (MgMP-HRP/NHA) in the Q_x band. The spectra were taken at various temperatures after hole burning within 7.3 cm^{-1} of the maximum of the spectral band at 5 K. (*b*) Evaluation of the temperature-cycling experiment. The hole area is plotted as a function of the cycling temperature by open circles and with the fitted solid line. A Gaussian distribution function (- - -) is generated from these data.

No correlation between Q_x and Q_y

Despite the fact that the resonant holes observed in the Q_y band are essentially broader than those burnt in the Q_x band, they are still narrower than the whole Q_y band by a factor of 6. This proves that the width of the Q_y band as well as that of the Q_x band is predominantly inhomogeneous. However, selective excitation (burning) in the Q_x band results in a broad nonresonant satellite hole in the Q_y band of width comparable to the entire inhomogeneous bandwidth. This result shows that energy selection through laser excitation in the Q_x electronic transition does not select a definite structural feature; that is, energy selection is not identical to site selection. Based on the results reported for the Q_x and Q_y bands of Mg-porphine in *n*-octane (Dicker et al., 1983), one can conclude that this effect may be similar in proteins and in other matrices. There seems to be a general consensus to the effect that the different electronic states of a molecule are not correlated (Lee et al., 1985). However, in the specific case of the Q_x and Q_y bands of tetrapyrroles, it should be mentioned that the opposite conclusion was reached for tetraphenylporphin in polystyrene (Lee et al. 1990).

The energy of Q-splitting

The energy separation of the Q_x-Q_y splitting is very sensitive to the exact structure of the matrix in which the chromophore is embedded; it increased by a factor of three upon binding the substrate and it decreased when the structure was slightly rearranged photochemically. Based on the x-ray structures of native HRP and of its complex with benzohydroxamic acid (Henriksen et al., 1998; Gajhede et al., 1997), the evident structural change upon substrate binding is the flipping of the side chain of the phenylalanine residue close to the heme and the presence of an additional water molecule in the heme pocket. The substrate does not seem to directly bind to the heme group. Additional studies are required to address the question of how these structural factors lead to the observed large splitting effect. In the previous Mg-porphine studies (Dicker et al., 1983; Platenkamp and Noort, 1982; Platenkamp, 1982), similar large differences in Q-splitting were observed in certain types of crystal sites in *n*-octane. Analogous effects were also reported for Zn-porphin (Canters and van der Waals, 1978; Shelby and MacFarlane, 1979). We believe that the symmetry of the electric field in the protein's heme pocket represents a crucial Q-splitting factor as suggested before (Laberge et al., 1998) and as theoretically modeled (Manas et al., 1999, 2000). Even a slight structural change, such as that occurring as a result of binding benzohydroxamic acid may lead to significant changes in the symmetry of this field by, for example, shielding or exposing certain groups of charged or dipole character. We suspect, however that changes in the ligation state of the metal-porphyrin may also have an even more significant effect on Q-splittings. The ligation of water was found especially very effective in causing Q_x-Q_y splitting in recent theoretical calculations reported for aluminum phthalocyanine tetrasulfonate (Reinot et al., 1999). The additional water molecule in the case of the substrate complex is a candidate likely to cause both charge distribution and ligational effects.

$Q_y \rightarrow Q_x$ relaxation

The $Q_y \rightarrow Q_x$ relaxation time of 1.5 ps for the NHA complex is on the short side of the range reported for the five

different sites of the Mg-porphine/*n*-octane system (Dicker et al., 1983). In that work, a negative correlation between the energy splitting and the relaxation time was found. It was suggested that the relaxation was mediated by phonon coupling as in the case of Zn-porphin in octane, which has a smaller Q-separation (Shelby and MacFarlane, 1979). In various hyperquenched glassy solvents a ~1-ps relaxation time from Q_y to Q_x was observed for Al-phthalocyanine (Reinot et al., 1999). In our previous study on vibrational relaxation in HRP the relaxation time varied in the range of 1–10 ps for different vibrational modes of pyrrole tautomers of the chromophore (Herenyi et al., 1998). We interpreted the variations based on the identification of the vibrational mode and its possible coupling to the phonon bath of the protein. This coupling may be different in the case of different tautomeric structures and also different for in-plane or out-of-plane modes. We did not observe any correlation between the magnitude of the vibrational energy and the time of relaxation.

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