Eur Biophys J (2000) 29: 429–438

ARTICLE

E. Balog · R. Galántai · M. Köhler
M. Laberge · J. Fidy

Metal coordination influences substrate binding in horseradish peroxidase

Received: 15 October 1999 / Revised version: 3 April 2000 / Accepted: 5 April 2000

Abstract To clarify the role of metal ion coordination in horseradish peroxidase C (HRPC), the effect of pressure and of an externally applied electric field on spectral holes was compared for both metal-free and Mg-mesoporphyrin-substituted horseradish peroxidase C (MP-HRP and MgMP-HRP), as affected by the binding of 2-naphthohydroxamic acid (NHA). The data are compared to earlier studies performed on the same derivatives. Results obtained for MP-HRP show the presence of a predominant MP tautomer, as well as that of another small population with different pocket field and isothermal compressibility (0.12 vs 0.24 GPa^{-1}). Binding NHA induces the formation of two new almost equal populations of MP-HRP tautomer complexes and the protein compressibility in both forms is increased to 0.50 and 0.36 GPa^{-1}. The protein structure becomes much softer than in the absence of NHA. Binding the same substrate to MgMP-HRP resulted in MgMP adopting a single conformation with no compressibility changes, while without NHA, two forms were possible. Stark effect results show charge rearrangement upon substrate binding in both cases. We propose that it is the presence of the metal that stabilizes the structure during the reorganization of the protein matrix induced by the substrate binding event. With the metal, only one conformation is adopted, without significant structural rearrangement but with charge redistribution. The dissociation constants determined for NHA binding to both derivatives and to native HRPC show that studies using mesoporphyrin and Mg-mesoporphyrin derivatives are relevant to investigating the specificity of the substrate-binding pocket in this enzyme.

E. Balog · R. Galántai · M. Laberge · J. Fidy (✉)
Institute of Biophysics, Semmelweis University of Medicine,
P.O. Box 2634, 1444 Budapest, Hungary
e-mail: judit@puskin.sote.hu

M. Köhler
Technische Universität München,
Lehrstuhl für Physik Weihenstephan, 85350 Freising, Germany

Key words Horseradish peroxidase · Spectral hole burning · Protein isothermal compressibility · Stark effect · Porphyrin Q-band splitting

Abbreviations *HRPC* isozyme C of horseradish peroxidase · *MP* mesoporphyrin IX · *MP-HRP* mesoporphyrin IX horseradish peroxidase C · *MgMP* · Mg(II)-mesoporphyrin IX · *MgMP-HRP* Mg(II)-mesoporphyrin IX horseradish peroxidase C · *NHA* 2-naphthohydroxamic acid · *SHB* spectral hole burning

Introduction

We have been studying the C isozyme of horseradish peroxidase (HRPC) for a long time. It contains a non-covalently bound heme group maintained in the protein matrix by a network of hydrophobic and electrostatic interactions (Yonetani et al. 1972). Our interest is not only motivated by the fact that it is representative of the important biochemical class of peroxidases, but also because it is a good model system to investigate the role of protein conformation in stabilizing a given – functionally important – structure (Fidy et al. 1998; Friedrich et al. 1994; Herenyi et al. 1995). HRPC is a good candidate because it binds such a wide variety of substrates that it is considered to defy all rules of enzyme specificity (Hewson and Hager 1979). As such, it then provides us with a protein system expected to undergo a variety of structural rearrangements to accommodate its different substrates (Fidy et al. 1989, 1992a). Specifically, we are interested in assessing the role – and the interactions – of the different elements making up the enzyme assembly. In a simple approach, we may consider the porphyrin prosthetic group with a central metal, embedded in a protein matrix that may acquire specific functionally significant populations of substates as a consequence of interactions with the porphyrin. The question is what interactions are important in this selection.

In our previous studies, we selected the laser excitation, low-temperature methods, spectral hole burning (SHB) and fluorescence line narrowing, to study the effect of substrate binding through the vibronic properties of a porphyrin at the heme site in HRPC. In this work, we report results of recent experiments that allow the comparison of mesoporphyrin IX horseradish peroxidase C (MP-HRP) and the magnesium-substituted enzyme (MgMP-HRP).

We compare the two systems on the basis of the isothermal compressibility of the protein, the protein-induced solvent shift, the vacuum absorption frequency of the chromophore, and the electric dipole moment change induced at the heme pocket, in the light of previous studies on the same derivatives.

The recent availability of a three-dimensional structure for horseradish peroxidase C (Gajhede et al. 1997) and of its complex with benzhydroxamic acid (Henriksen et al. 1998) has provided a clear picture of the aromatic structural arrangement of the substrate binding site at the edge of the heme. It had been long proposed to be common characteristics of plant peroxidases that a hydrogen-bond network exists around the active site, provided by specific invariant and catalytically relevant residues, such as Arg38, Phe41, His42, Asn70, His170, Phe221 and Asp247 (Smulevich 1998). While we now have a very good understanding as to how the heme pocket residues biochemically control the catalytic reaction mechanism and binding process of several substrates (Ortiz de Montellano et al. 1995; Veitch 1995a, b), the advantage of recently obtaining the X-ray crystallographic structural information on the enzyme-substrate complex had not been fully exploited. The fact that the respective X-ray structures show no significant heme distortions as a result of substrate binding prompted us to refocus our earlier studies on investigating the role of metal coordination.

In this work, we report on comparing HRPC in the case when the heme is substituted for a metal-free porphyrin (MP) to that of metal coordination (Mg-MP). We present the dissociation constants (K_d) for NHA binding to native HRPC and to the substituted forms and show that the study is relevant to the native system since the substrate-binding capacity is only slightly modified.

Studies on metallo-substituted and heme-modified peroxidases have also yielded a wealth of important structure-function information (Dunford 1991), but the question as to whether the geometry determined by the protein matrix modulates the metal-prosthetic group properties or whether the metal itself modulates the molecular geometry of the binding site (and by extension, the reaction and binding processes) has yet to be specifically addressed. This is the other reason motivating our choice of metal-containing and metal-depleted derivatives. It becomes even more relevant if we bear in mind that the functionally significant conformations of hemoglobin and cytochrome *c* have been shown to be primarily determined by the interaction of the protein matrix with its prosthetic group and not with the heme iron (Sano 1979).

We will show that the substrate-binding effect is totally different in the two systems and that the results allow us to comment on the role of the metal in specifying the architecture of the protein around the porphyrin upon binding an aromatic donor.

Materials and methods

Sample preparation

HRPC was isolated and purified from horseradish. The native heme was removed from the enzyme using the acid methyl ethyl ketone method (Teale 1959). The apoprotein was reconstituted with mesoporphyrin IX or with the Mg(II) mesoporphyrin disodium salt from Porphyrin Products (Logan, Utah, USA) dissolved in ethanol. For the K_d titrations, micromolar concentrations of the enzymes were prepared in 50 mM ammonium acetate buffer, pH 5.1, or 50 mM phosphate buffer, pH 7.0. Solid 2-naphthohydroxamic acid (NHA) was dissolved in methanol (Merck, spec grade) and used as a 20% (v/v) MeOH-H_2O solution. For the low-temperature P-tuning and SHB measurements, the enzymes were prepared at ca. 400 μM in 50 mM ammonium acetate buffer, pH 5.0, or in phosphate buffer, pH 7.0, containing 50% v/v glycerol to ensure good optical quality glass formation at the experimental cryogenic temperatures. The complexes of MP-HRP and MgMP-HRP with substrate were prepared in the same buffers with a ~5-fold excess of NHA. Concentrations were calculated using the following extinction coefficients (lab determinations): for native HRPC in 20 mM phosphate buffer, pH 6.04, $\varepsilon_{403} = 94.21$ mM^{-1} cm^{-1}; for MgMP-HRP, $\varepsilon_{414} = 108.21$ mM^{-1} cm^{-1}; for MP-HRP, $\varepsilon_{400} = 150.08$ mM^{-1} cm^{-1}; and for 2-NHA in 20% (v/v) MeOH-H_2O $\varepsilon_{280} = 7.04$ mM^{-1} cm^{-1}.

K_d determinations

The K_d titrations were performed at room temperature (RT) for native HRPC under the same conditions as previously reported in binding experiments with naphthohydroxamic acids and the native enzyme (~2 μM, 20 mM ammonium acetate, pH 6.04) (Schonbaum 1973). The measurements were carried out on a Cary-Varian 4E spectrophotometer by monitoring absorbance at 410 nm, where no uncomplexed NHA absorbs and where good, linear measurements are possible (λ_{max} HRPC = 407.5 nm, λ_{max} HRPC-NHA = 403 nm). The K_d titrations of the derivatized HRPC were performed in the fluorescence emission mode, because absorption changes upon substrate binding are only of the order of 1.5–2% absorbance units. Fluorescence measurements also allow work at much lower derivative concentrations (0.5–0.9 μM) with substrate binding easily detectable under Soret excitation. Also, uncomplexed NHA does not fluoresce. These measurements were performed on an Edinburgh Analytical Instruments CD900 luminometer equipped with a 75-W Xe lamp. Excitation and emission slits were set at 1 nm and a temperature control unit was used to maintain the sample at 22 °C. At RT in 50 mM ammonium acetate, pH 5.1, MP-HRP has emission maxima at 615 and 679 nm and Soret excitation maximum at 400 nm. MgMP-HRP has emission maxima at 588 and 642 nm and Soret excitation maximum at 414 nm. The titrations were accordingly performed using 400 nm excitation for MP-HRP, with emission monitored at 613 nm, and using 414 nm excitation for MgMP-HRP with emission monitored at 584 nm.

Spectroscopic techniques

Low-temperature absorption spectra were acquired with a Jobin-Yvon monochromator THR 1000 (Jobin-Yvon, Longjumeau, France) at a resolution of 2 cm^{-1}. SHB was performed at 1.7 K with the sample inserted in a He bath cryostat using a single-frequency ring dye laser. The spectral width of the laser was of the order of 1 MHz. Burning power and burning times were about 100 μW and 30 s, respectively. The spectral line corresponding to the electronic transition at the energy of exciting laser light was determined in the form of a spectral hole, detected in transmission mode with a 100 times attenuated intensity after burning. During the P-tuning experiment, the samples were kept at 1.7 K. Pressure was transmitted and regulated via He gas with an accuracy of 10^{-3} MPa (0.010 bar) and varied by increments up to 1.1 MPa (11 bar). Samples were sealed in small plastic bags to ensure isotropic pressure conditions. The pressure cell was immersed in liquid helium. For the Stark effect experiments the samples were sealed in glass cuvettes, placed between two plane electrodes and kept at 1.7 K. The applied field strength E_0 was varied between 0 and 21.7 kV/cm. The laser polarization was either parallel or perpendicular to the Stark field.

P-tuning hole burning spectroscopy

The basic principles as well as applications of P-tuning spectroscopy for the study of proteins have recently been fully reviewed (Fidy et al. 1998; Köhler et al. 1998). The term "P-tuning" reflects the fact that in the present studies the effect is measured in the range of low pressure changes (i.e. up to 2.5 MPa or 25 bar). Consequently, the spectral changes are also very small and can only be detected by narrow spectral lines produced in the SHB method. In an electronic transition, the inherent homogeneous spectral linewidth is theoretically related to the excited state lifetime according to the time-dependent perturbation theory. The narrower the line, the longer the excited state lifetime. In our chromophores, the S_1 electronic excited state has lifetimes of about 20 ns (MP) or 2 ns (MgMP). The respective experimental homogeneous spectral line width at the temperature of the experiment (including optical dephasing and spectral diffusion) is in the range of 100–1000 MHz (Schlichter et al. 2000). This was conveniently measurable as a "narrow" hole in the present experiments. The effect of changing the external pressure is that spectral lines may shift, broaden or even split. A simple model to describe the pressure-induced line shift (s) of a spectral hole was elaborated by Laird and Skinner (1989) and subsequently modified for hemeprotein systems (Fidy et al. 1992a, b; Friedrich et al. 1994; Gafert et al. 1994; Zollfrank et al. 1991a). According to the model, this shift is directly proportional to the solvent shift which arises from the matrix of the chromophore. In our experiments, this matrix is equivalent to the protein. Supposing that the interaction of matrix and chromophore can be described by the attractive part of the Lennard-Jones potential, then the shift of the transition frequency monitored by the spectral hole is:

$$s = 2\kappa v_s \Delta p \tag{1}$$

where κ is the isothermal compressibility of the matrix, Δp is the pressure change, and v_s the solvent shift, defined as:

$$v_s = v_b - v_{vac} \tag{2}$$

where v_b is the burning frequency and v_{vac} is the vacuum absorption frequency of the chromophore. By plotting $s/\Delta p$ versus the burning frequency v_b, two quantities can be extracted from linear fits of the data: the slope yields the isothermal compressibility κ of the chromophore environment, i.e. of the protein, and the frequency at which the pressure shift $s/\Delta p$ vanishes is called the vacuum frequency v_{vac}.

The isothermal compressibility (κ) is of special interest because it is very difficult to measure by conventional methods such as densimetry and ultracentrifugation and because, unlike the adiabatic compressibility β, usually measured by sound velocity measurements, it is directly related to the volume fluctuation of a protein (δV) (Cooper 1976):

$$\delta V^2 = kTV\kappa \tag{3}$$

The vacuum frequency (v_{vac}) is the one at which the chromophore would absorb if not subjected to its environment and it thus may be different from the actual transition frequency. As such, it is then indicative of optical center/matrix interactions (Fidy et al. 1998; Köhler et al. 1998), and its absolute value is influenced by the in situ conformation of the prosthetic group in the protein.

The two quantities that can be determined thus reflect different properties of the protein ensemble: the vacuum frequency v_{vac} depends on the chromophore conformation – induced by the protein – while the isothermal compressibility κ depends directly on that of the protein.

Spectral holes in external electric fields

The consequence of the Stark effect is that a spectral hole subjected to an externally applied field $\boldsymbol{E}_0$ shifts by a frequency difference Δv:

$$h\Delta v = -f\Delta\vec{\mu}\boldsymbol{E}_0 - \frac{1}{2}f^2\boldsymbol{E}_0\Delta\hat{\alpha}\boldsymbol{E}_0 \tag{4}$$

where h is Planck's constant, $\Delta\vec{\mu}$ the difference of the dipole moments between the excited state $\vec{\mu}_e$ and the ground state $\vec{\mu}_g$:

$$\Delta\vec{\mu} = \vec{\mu}_e - \vec{\mu}_g \tag{5}$$

and $\Delta\hat{\alpha}$ is the difference of the polarizability tensors in the respective states and f is a field correction factor (Koehler and Woehl 1995).

The dipole moment difference $\Delta\vec{\mu}$ that determines the behavior of the sample in an external electric field may originate from two sources: (1) there may be a molecular dipole moment difference $\Delta\vec{\mu}_0$ determined by the chromophore and/or (2) there may be an induced dipole moment difference $\Delta\vec{\mu}_i$ generated by the matrix. Generally, the local fields experienced by chromophores and generated by the surrounding matrix are significantly larger than externally applied electric fields. Therefore, the quadratic term in Eq. 4 can be neglected. The observed spectral features are therefore linear in $\boldsymbol{E}_0$:

$$h\Delta v = -f\Delta\vec{\mu}\boldsymbol{E}_0 \tag{6}$$

According to Eq. (6), the hole splitting depends on the dipole moment difference (either intrinsic or matrix induced) of the chromophore. How this splitting can be detected depends on the angle γ between the transition dipole moment $\vec{\mu}_{ge}$ and the dipole moment difference $\Delta\vec{\mu}$:

1. For $0° < \gamma < 55°$, the hole splits if the polarization of the laser field is parallel to the external electric field.
2. For $55° < \gamma < 90°$, the hole splits if the laser field is perpendicular to the external field.
3. For γ close to 55°, the hole does not split under any polarization direction. The same is observed in the case of inversion symmetry.

Stark effect measurements on spectral holes thus provide information about the local electric field experienced by the chromophore. Our previous work has shown that the hemes of both MP-HRP and MgMP-HRP have an effective inversion symmetry in a glassy matrix (Köhler et al. 1997) and thus their molecular dipole moment is very small. Therefore, the behavior of these chromophores in proteins in a Stark experiment reflects the dipole moment of the chromophore induced by the protein matrix (Fidy et al. 1998; Köhler et al. 1998). Hence, the Stark effect hole-burning experiment yields information about the field experienced at the heme and, by extension, about the matrix imposing the field.

Results

K_d determinations

We determined the K_d values for NHA binding to native HRPC and both derivatives to characterize how suitable the substituted enzymes are as HRP models. The dissociation constants were calculated using the following equation: $1/\Delta I = (K_d/\Delta I^{\infty})\ (1/S) + (1/\Delta I^{\infty})$, where S is the NHA concentration, ΔI is the observed absorption or fluorescence emission change at the monitoring wavelength and ΔI^{∞} is the spectral change at saturation (Schejter et al. 1976). A typical titration is shown in Fig. 1 and the K_d values are listed in Table 1. In the case of MP-HRP, substrate binding shifts the 615 nm emission to 622 nm and the 588 nm emission of MgMP-HRP is similarly shifted to 584 nm. The clear isosbectic points are also indicative of only two species in solution.

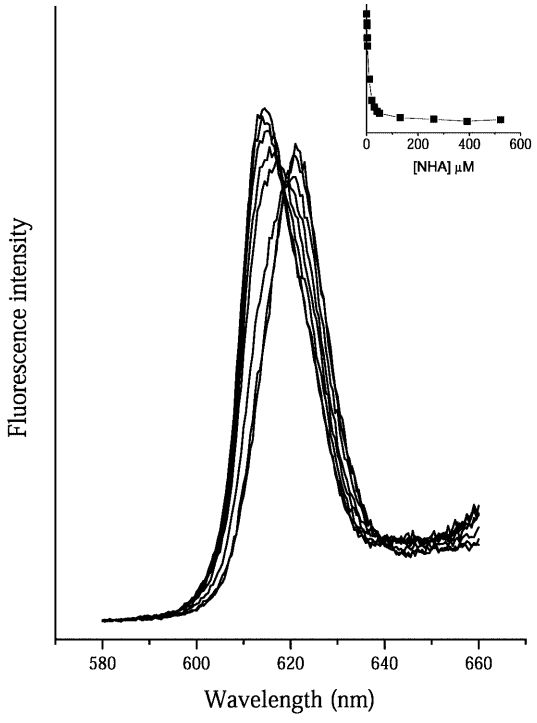

Fig. 1 Fluorescence emission titration of 1.5 μM MP-HRP with 2-naphthohydroxamic (NHA) acid in 50 mM ammonium acetate buffer, pH 5.1. The *inset* shows the K_d titration monitoring fluorescence intensity decay at 613 nm as a function of increasing NHA concentration. The K_d is 6.8 μM; see Table 1

Table 1 Dissociation constants for the binding of NHA to native HRPC, MP-HRP and MgMP-HRP

Enzyme	K_d (μM)
HRPC	0.5
	0.2[a]
MP-HRP	6.8
MgMP-HRP	4.0

[a] Schonbaum et al. (1973)

The binding of NHA to both MP-HRP and MgMP-HRP was found to be strong, although somewhat less strong than in the case of the native enzyme (4.0 μM for MgMP-HRP versus 0.5 μM for the native form). The difference is less pronounced, however, than what was observed in the binding of the 1- and 2-naphthohydroxamic acids to HRPC: the reported K_d values are 20 and 0.2 μM, respectively (Schonbaum 1973). The K_d values which we report for the derivatives thus fall into the range of dissociation constants reported for the binding of aromatic substrates to the native enzyme. On the one hand, this strengthens the consensus view that access to the active site of the peroxidase is controlled by a finely tuned network of nonbonded interactions (Henriksen et al. 1998), which we believe is what the different K_d values reported for the binding of the native enzyme to substrates of similar chemical structure reflect. On the other hand, they show that our derivatization procedure yields an HRPC analog which binds the substrates of the native enzyme without the K_d values being indicative of a severe derivatization-induced perturbation of the protein structure.

At this point, we should ask which of the derivative substitutions, i.e. meso for protoporphyrin versus Mg for Fe, is more likely to be responsible for the observed difference in association constants reported for the native enzyme and the derivatives? When compared to the K_d value obtained for metal-free MP-HRP (7.0 μM), we note that the substrate binds slightly more strongly to the metal derivative by a factor of two, which we interpret as indicative of a subtle role for the metal in the substrate-binding event. The insertion of a mesoporphyrin – instead of a protoporphyrin – is known to have no effect on the enzyme activity or on the formation of the catalytic intermediates, i.e. compounds I, II and III. Meso- and protoporphyrin differ in that the former has an ethyl substituent at positions 2 and 4 of the ring, while the latter has a vinyl substituent at the same positions. The catalytically important substituent groups have been identified as the propionic acid chains at positions 6 and 7, conserved in both meso- and protoporphyrins, and it is only when these substituents are removed or modified that enzyme functionality is lost (Hewson and Hager 1979). The only candidate then left to account for the slightly higher affinity of MgMP-HRP for the substrate is the metal itself.

P-tuning experiments in the uncomplexed peroxidases

The overall P-tuning results for the uncomplexed samples are summarized in Table 2. Figure 2a and b presents the results obtained for MP-HRP at pH 5.1. The absorption spectrum under the present conditions in the $Q_{(0,0)}$ range consists of one predominant band, B1, and of a second, very small one, B3 (Fig. 2a). The labeling of the two bands in Fig. 2a follows the labeling scheme used in previous studies at different pH values and in photochemical studies in which other tautomer states

Table 2 P-tuning and Stark effect SHB results in MP-HRP and MgMP-HRP

Sample	ν_{vac} (cm^{-1})	κ (Gpa^{-1})	Polarization of hole splittings	γ
MP-HRP (B1)	16,620	0.12	Parallel	45°
MP-HRP (B3)	16,270	0.24	Perpendicular	70°
MgMP-HRP (Q_{x1})	17,560	0.11[a]	Perpendicular[b]	75°
MgMP-HRP (Q_{x2})	17,850	0.11[a]	No splitting[b]	~55°

[a] Balog et al. (1997)
[b] Köhler et al. (1997)

Table 3 P-tuning and Stark effect SHB results in MP-HRP, MgMP-HRP complexed to NHA

Sample + NHA	ν_{vac} (cm^{-1})	κ (GPa^{-1})	Polarization of hole splittings	γ
MP-HRP (B1′)	16,340	0.36	Perpendicular	70°
MP-HRP (B2′)	16,410	0.50, 0.4[a]	No splitting	~55°
MgMP-HRP (Q_x)	17,655	0.13[b]	No splitting	~55°
MgMP-HRP (Q_y)	No holes			

[a] Fidy et al. (1992a)
[b] Balog et al. (1997)

(B2, B4) could also be populated (Fidy et al. 1992b). The triangles show the results of the P-tuning hole burning experiments. Similar data were presented earlier for pH 8 conditions (Friedrich et al. 1994; Gafert et al. 1994). We also reported that the MP tautomer distribution in the sample depends on the pH (Fidy et al. 1987; Gafert et al. 1994). It is seen in Fig. 2a that narrow holes could be burnt in both bands, which allows their assignment as $S_0 \rightarrow S_1$ transitions. The efficiency of the hole burning – in the case of similar excitation probabilities – characterizes the population of chromophores within the inhomogeneously broadened bands; this agrees well

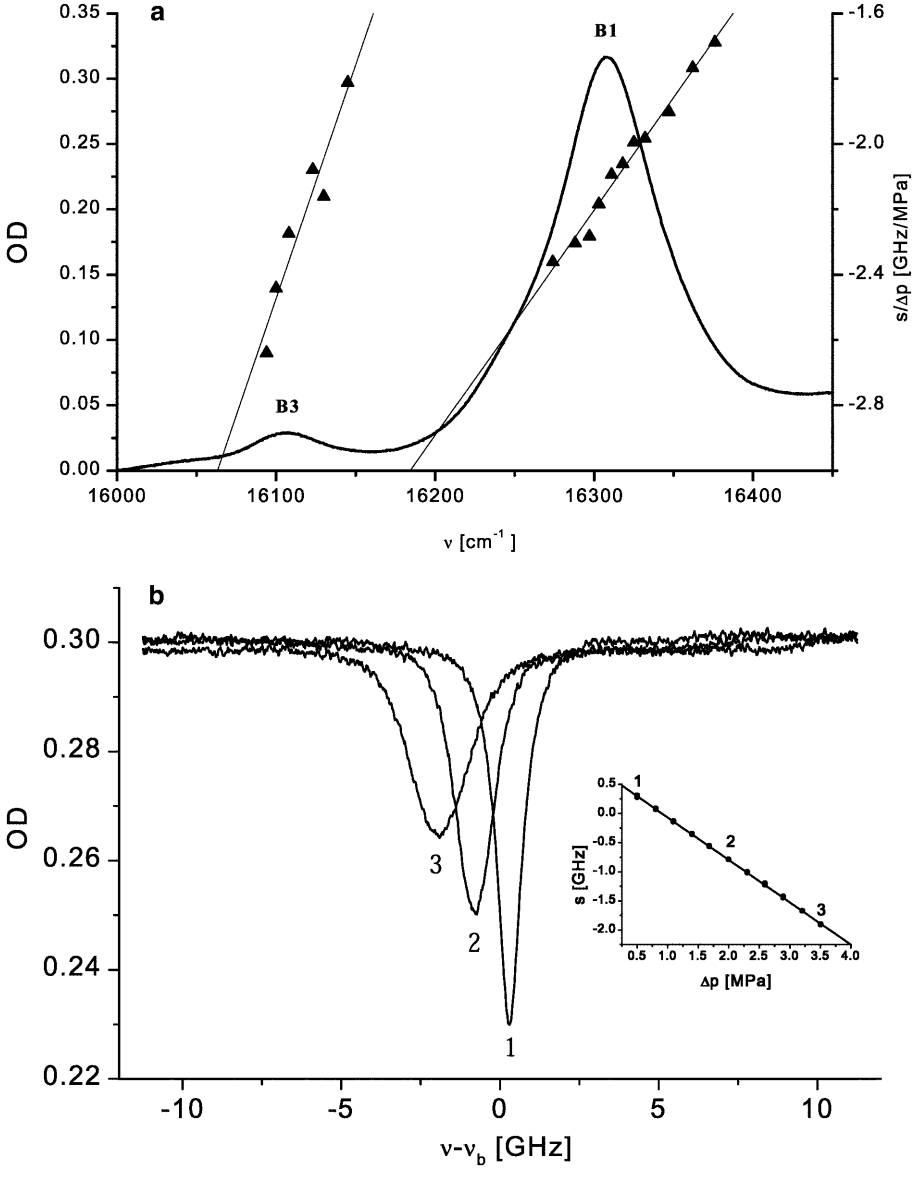

Fig. 2a Frequency shift per unit pressure as a function of burning frequency in MP-HRP overlaid on the absorption spectrum of the sample. The *straight lines* are least-squares fits to the data plotted as $s/\Delta p$ vs. burning frequency ν_b (see text) and their slope yields 2κ. For B1, $\kappa = 0.12$ GPa^{-1}, $\nu_{vac} = 16{,}620$ cm^{-1}; for B3, $\kappa = 0.24$ GPa^{-1}, $\nu_{vac} = 16{,}270$ cm^{-1}. $T = 1.7$ K; sample concentration was ~400 μM in 50 mM ammonium acetate, pH 5.1, 50% glycerol. **b** Experimental data obtained in a MP-HRP P-tuning experiment at a burning frequency of 16,303 cm^{-1}, showing the pressure shift of three holes: *1* with $\Delta p = 0.53$ MPa, *2* with $\Delta p = 1.92$ MPa and *3* with $\Delta p = 3.53$ MPa. *Inset*: linearity of spectral shift (s) as a function of Δp

with the relative intensities of bands B1 and B3. The shift of spectral holes could be well measured in both bands and a linear effect with Δp was observed (cf. Fig. 2b, inset). The shift per pressure as a function of the burning frequency is also linear in both bands (triangles) and the corresponding compressibilities and vacuum frequencies are significantly different: $\kappa = 0.12$ GPa^{-1}, $\nu_{vac} = 16{,}620$ cm^{-1} versus $\kappa = 0.24$ GPa^{-1}, $\nu_{vac} = 16{,}270$ cm^{-1}. At pH 8, three tautomer forms (bands) were found populated, out of which the state B1 was also predominant. The compressibility measured in bands observed at comparable positions was also similar under the two pH conditions.

The P-tuning results previously obtained for MgMP-HRP – also at pH 5 – (cf. Table 2 and Balog et al. 1997) were indicative of two MgMP-HRP populations characterized by different vacuum frequencies (17,560 cm^{-1} and 17,850 cm^{-1}, respectively); thus they also correspond to two different prosthetic group conformations. These obviously cannot be pyrrole tautomers. We proposed that they were conformations with different ligation states. In MgMP-HRP, the same compressibility values were obtained in the two bands ($\kappa = 0.11$ GPa^{-1}), similar to the values obtained for B1 in MP-HRP.

Stark effect SHB experiments in the uncomplexed peroxidases

Figure 3 presents the Stark effect SHB results obtained for MP-HRP with holes burnt in the B1 and the B3 bands with the laser polarization either parallel or perpendicular to the Stark field. Similar results at pH 8 and after photochemical transformation were reported earlier (Gafert et al. 1995). Under parallel polarization, holes burnt in the B1 band show a rather small splitting and no splitting under perpendicular polarization. The earlier measurements on the B1 band at pH 8 were performed up to 12 kV/cm. No splitting was observed under either polarization. We believe that the present results at pH 5 with only a small splitting induced at significantly higher voltages under parallel polarization may be indicative of a similar effect. The observed splitting now allows us to estimate the angle γ between the transition dipole moment and the dipole moment difference as lying between 0 and 55°; it was calculated to be equal to 45°. The holes burnt in the B3 band clearly show the opposite splitting pattern, i.e. a splitting under perpendicular polarization, yielding a γ angle equal to 70°. A similar polarization effect and a γ value of 63° was found at pH 8 in the tautomer form of B2 at 16,200 cm^{-1} (Gafert et al. 1995).

Results of comparable experiments at pH 5 were reported for MgMP-HRP (Köhler et al. 1997). Hole splitting was observed in the predominant 17,068 cm^{-1} band under perpendicular polarization. Here, γ is calculated to be 75°. No splitting was observed for the holes burnt into the other band at 17,160 cm^{-1}, whether under perpendicular or parallel polarization, and this is indicative of a γ angle close to 55°, or of the case of inversion symmetry (cf. Table 2).

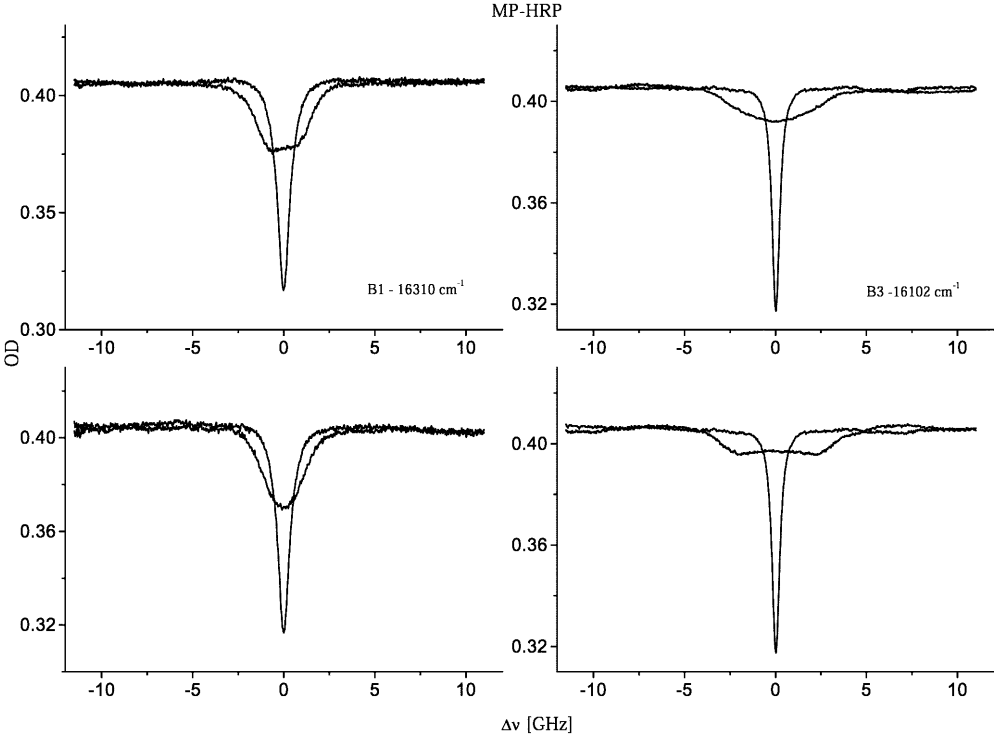

Fig. 3 Spectral holes burnt into the B1 (*left*) and B3 (*right*) bands of MP-HRP, ca. 400 μM in 50 mM ammonium acetate, pH 5.1, 50% glycerol, under the influence of the Stark field (22 kV/cm) under ∥ (*top*) and ⊥ polarization (*bottom*)

P-tuning experiments in the peroxidases with bound NHA

In further experiments, NHA was added to MP-HRP and also compared to the results obtained for MgMP-HRP under the same conditions (Balog et al. 1997). The complexed form has significantly different absorption spectra in both cases. The P-tuning results obtained for MP-HRP + NHA are shown in Fig. 4 and summarized in Table 2. The spectrum of the complex in this region consists of two bands, labeled B1′ and B2′. Thus, after substrate binding, two new populations of chromophore configurations of comparable populations exist. We performed photochemical transformation on the bands and studied the temperature effect on ground state reconversion. Above 100 K, the tautomers could be reconverted from one form to the other (results not shown). Based on previous knowledge about the height of barriers between the ground state forms of a variety of MP tautomers that can be stabilized within the heme pocket of HRP (Fidy et al. 1992b; Herenyi et al. 1995; Zollfrank et al. 1991b), we concluded that B1′ and B2′ corresponds to the two forms with opposite pyrrole nitrogens protonotated (linear tautomers, cf. Voelker et al. 1977). We reported earlier P-tuning data at pH 5 for B2′ (Fidy et al. 1992a), but B1′ was not investigated. The more extensive present P-tuning experiments confirmed the earlier results in both forms: compared to the uncomplexed proteins, we observed a significant increase in compressibility in both tautomer states of the complex. In Fig. 4, the triangles show the results of the P-tuning experiments in the linear range. The compressibility in B1′ is $\kappa = 0.36$ GPa^{-1} and in B2′ it is $\kappa = 0.50$ GPa^{-1}. The conclusion is that the fit of the mesoporphyrin tautomer in the heme pocket is much looser in the complex than that in the predominant B1 form of the uncomplexed protein, and it is also looser than that in the secondary B3 population. In the case of the MgMP-HRP complex bound to NHA, the P-tuning experiment proved that the absorption spectrum is indicative of only a single 0,0 transition band and thus of a single structural configuration of the chromophore in the complexed form. The κ value of 0.13 GPa^{-1} was very similar to that in the uncomplexed form and the vacuum frequency of the single conformation was between the two values of the two forms possible without the substrate (Balog et al. 1997).

Stark effect SHB experiments in the peroxidases bound to NHA

Stark effect experiments were performed in the present study on both complexed samples (cf. Table 2). The results are shown in Fig. 5. In the case of MP-HRP + NHA, we observe an unambiguous splitting under perpendicular polarization, indicative of a γ angle lying between 55 and 90°. In the B2′ band of MP-HRP, the hole does not split under either polarization position, which points to a γ angle close to 55° or inversion symmetry (results not shown). In the case of MgMP-HRP + NHA, no splitting was observed, neither under parallel nor under perpendicular polarization.

Discussion

Comparison of MP-HRP and MgMP-HRP in the absence of NHA

The P-tuning results obtained for MP-HRP demonstrate that different chromophore tautomers – characterized by different vacuum frequencies (0.12 GPa^{-1} for B1 and 0.24 GPa^{-1} for B3) – are stabilized by proteins with different compressibilities. The higher value of κ in the

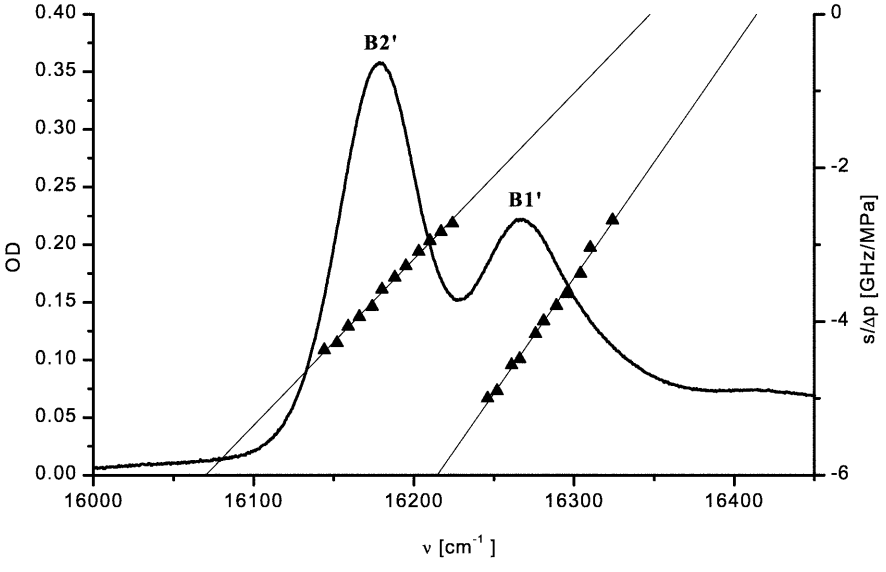

Fig. 4 Frequency shift per unit pressure as a function of burning frequency in MP-HRP complexed to NHA. For the B1 band, $\kappa = 0.36$ GPa^{-1}, $\nu_{vac} = 16{,}340$ cm^{-1}; for the B2 band, $\kappa = 0.50$ GPa^{-1}, $\nu_{vac} = 16{,}410$ cm^{-1}. $T = 1.7$ K

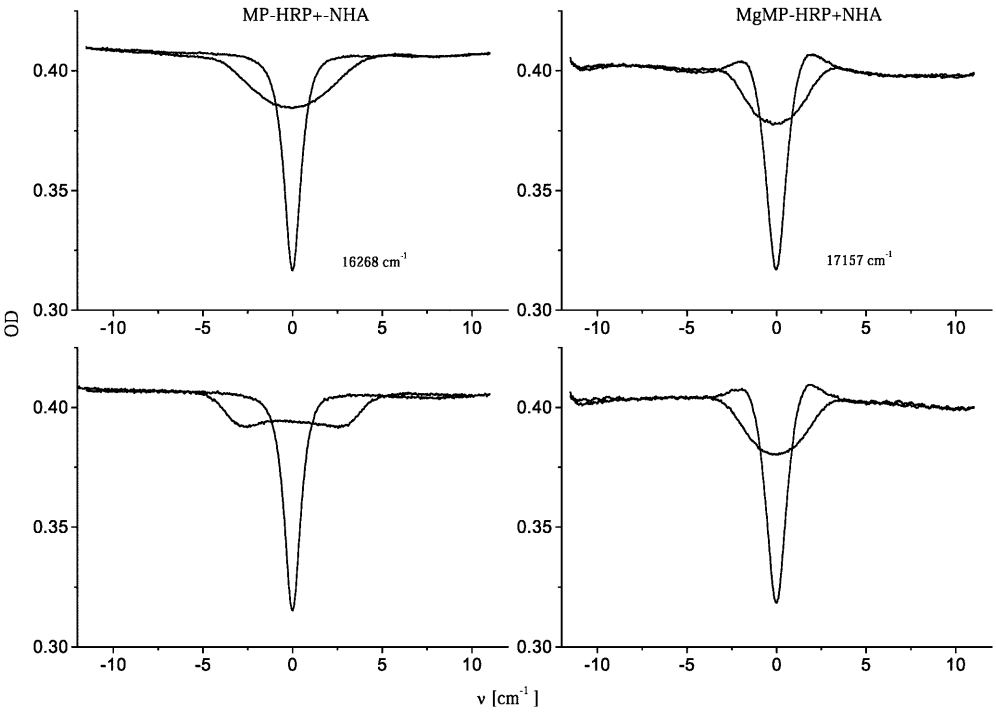

Fig. 5 *Left*: spectral holes burnt into the B1 band of MP-HRP complexed to NHA at 16,268 cm^{-1}, under the influence of the Stark field (22 kV/cm) under ∥ (*top*) and ⊥ (*bottom*) polarization. *Right*: spectral holes burnt in the higher energy band of MgMP-HRP complexed to NHA at 17,157 cm^{-1}, under the influence of the Stark field (22 kV/cm) and under ∥ (*top*) and ⊥ (*bottom*) polarization

case of the small population of B3 shows a looser fit of this spectral form to the protein.

In the case of MgMP-HRP, two spectral populations with different vacuum frequencies are also distinguishable but their compressibilities are the same (cf. Table 2). This suggests that these two metalloporphyrin spectral forms are stabilized by very similar protein conformations. We note here that this κ-value of 0.11 GPa^{-1} is identical to the value obtained for MP-HRP for the B1 form, which supports the idea that the predominant mesoporphyrin structural form in MP-HRP is well equilibrated with the protein. However, it raises the question as to whether the protein conformation in species with similar compressibilities are absolutely identical, or if the respective amino acid arrangements of the heme pocket are different to an extent which does not affect the compressibility. We turn to the Stark effect hole-burning results to elucidate this point. The very different hole-splitting patterns recorded for MP-HRP and MgMP-HRP (cf. Table 2) are indicative of different electrostatic environments for the respective optical centers and we propose that the protein conformations are then not the same in spite of their similar κ values. Recently, we reported on different c-type cytochromes sampling "electrostatic" conformational space with markedly different potentials being experienced at the heme (Laberge et al. 1999). These results showed that conformational changes can be thought of as consisting of charge redistributions as much as of structural rearrangements and we believe that the Stark results are indicative of the same type of conformational phenomenon here.

Effect of NHA binding on metal-free and Mg derivatives

Substrate binding has a major effect on the spectral properties of both derivatives. After addition of NHA to MP-HRP, still more than one prosthetic group conformation exists but they are different from those of B1 and B3 (Fig. 4). The almost identical integrated optical density of the two new spectral bands shows that the protein structure does not strictly select tautomers on the basis of specific H-bonding patterns involving the hydrogens of the protonated nitrogens. The temperature effect studies (Fidy et al. 1992b) suggest that these two forms are the well-known two linear forms found in Shpol'skii matrices (Voelker et al. 1977). The compressibility significantly increases, i.e., the protein around the porphyrin becomes much softer. Use of Eq. (3) with $V = 10^5$ Å allows us to estimate protein equilibrium volume fluctuations that at least double from the uncomplexed state (with $\kappa = 0.12$ GPa^{-1} for B1) to the complexed state (with $\kappa = 0.36$ GPa^{-1} for B1′ or 0.50 GPa^{-1} for B2′). The relative protein matrix charge distribution also changes considerably, as shown by the Stark SHB results (Fig. 5).

The P-tuning data on MgMP-HRP showed that, as a result of NHA binding, the peroxidase becomes "committed" to a single prosthetic group conformation. The protein compressibility is only slightly increased relative to the uncomplexed form of the enzyme, i.e. the protein matrix does not undergo a significant structural change. Substrate binding, however, does result in charge reorientation in the heme pocket, as inferred by the data of the Stark experiments presented here and in earlier

results that showed an increased energy separation of the split Q-energy level and shift of the spectral origins (Balog et al. 1997). It seems that it is this charge reorientation, much more than the spatial rearrangements reflected in κ, that "locks" the metal-containing peroxidase into a single conformation upon substrate binding. The Stark effect SHB results for both metal-free and metal-bound peroxidases are indicative of protein charge reorganization near the prosthetic group, as reflected by the contrasting hole splitting patterns: if the angle between the transition dipole moment and the dipole moment difference varies, this can only be due to variations in the electric field experienced at the heme (Köhler et al. 1996; Laberge 1998; Laberge et al. 1998). What is of interest is that, in the presence of metal coordination, the different prosthetic group populations are not associated with a change of the overall compressibility of the protein but only with different matrix-induced fields. In contrast, in the absence of metal the different prosthetic group populations are stabilized by totally different structural (as reflected by the different κ values) and electrostatic protein environments.

Our work suggests that HRPC is then capable of sampling "electrostatic" – rather than "structural" substates – and that it adopts a single, preferred conformation upon substrate binding. The peroxidase selects this single conformation only in the presence of metal ion coordination.

Conclusions

In this study, we investigated the significance of metal coordination to the prosthetic group of HRPC. New P-tuning and Stark effect SHB measurements performed on MP-HRP were compared to results obtained earlier on MgMP-HRP. They show that metal coordination has a stabilizing effect on the conformation of the porphyrin-protein structure. In the uncomplexed state, both the metal-free and metal-coordinated enzymes show different conformations of prosthetic groups. Upon adding substrate in the presence of metal ion coordination, a well-defined prosthetic group conformation becomes stabilized by the enzyme and the compressibility does not change. Spectral properties, however, show charge redistribution around the porphyrin. In contrast, substrate binding in the absence of metal coordination induces the formation of new varieties of porphyrin tautomer conformations, the coupling of which to the protein becomes structurally less determined.

Acknowledgements The authors thank Prof. J. Friedrich for kindly providing them with the possibility of performing spectral hole burning experiments at Lehrstuhl für Physik Weihenstephan, Technische Universität München, Freising, Germany, as well as the following agencies for support: the German-Hungarian WTZ program (J.F., E.B.), the Fond der Chemischen Industrie (M.K.), the "Structural Biochemistry" Ph.D. program (Eötvös University, Budapest) (E.B.). M.L. gratefully acknowledges support from the Hungarian postdoctoral grant MKM FKFP 1191/1997 (J.F.) and from the joint NSF-MTA International cooperative grant (MTA 122 and NSF 9812324).

References

Balog E, Kis-Petik K, Fidy J, Köhler M, Friedrich J (1997) Interpretation of multiple $Q_{(0,0)}$ bands in the absorption spectrum of Mg-mesoporphyrin embedded in horseradish peroxidase. Biophys J 73: 397–405

Cooper A (1976) Thermodynamic fluctuations in protein molecules. Proc Natl Acad Sci USA 73: 2740–2761

Dunford BH (1991) Horseradish peroxidase: structure and kinetic properties. In: Everse I, Everse KE, Grisham MB (eds) Peroxidases in chemistry and biology, vol 2. CRC Press, Boca Raton, pp 1–24

Fidy J, Paul K-G, Vanderkooi JM (1987) The pH dependence of phototautomerization in horseradish peroxidase monitored by fluorescence line narrowing spectroscopy. Chem Phys Lett 142: 562–566

Fidy J, Paul KG, Vanderkooi J (1989) Differences in the binding of aromatic substrates to horseradish peroxidase revealed by fluorescence line narrowing. Biochemistry 28: 7531–7541

Fidy J, Vanderkooi J, Zollfrank J, Friedrich J (1992a) Softening of the packing density of horseradish peroxidase by a H-donor bound near the heme pocket. Biophys J 63: 1605–1612

Fidy J, Vanderkooi JM, Zollfrank J, Friedrich J (1992b) More than two pyrrole tautomers of mesoporphyrin stabilized by a protein. A site selection spectroscopic study. Biophys J 61: 381–391

Fidy J, Balog E, Köhler M (1998) Proteins in electric fields and pressure fields: experimental results. Biochim Biophys Acta 1386: 289–303

Friedrich J, Gafert J, Zollfrank J, Vanderkooi J, Fidy J (1994) Spectral hole burning and selection of conformational substates in chromoproteins. Proc Natl Acad Sci 91: 1029–1033

Gafert J, Friedrich J, Vanderkooi J, Fidy J (1994) Correlation between protein conformation and prosthetic group configuration as tested by pH effects: a hole burning study on mesoporphyrin-IX substituted horseradish peroxidase. J Phys Chem 98: 2210–2214

Gafert J, Friedrich J, Vanderkooi J, Fidy J (1995) Structural changes and internal fields in proteins: a hole-burning Stark effect study of horseradish peroxidase. J Phys Chem 99: 5224–5227

Gajhede M, Schuller DJ, Henriksen A, Smith AT, Poulos TL (1997) Crystal structure of horseradish peroxidase C at 2.15 Å resolution. Nat Struct Biol 4: 1032–1038

Henriksen A, Schuller DJ, Meno K, Welinder KG, Smith AT, Gajhede M (1998) Structural interactions between horseradish peroxidase C and the substrate benzhydroxamic acid determined by X-ray crystallography. Biochemistry 37: 8054–8060

Herenyi L, Fidy J, Gafert J, Friedrich J (1995) Energy landscape of the tautomer states of mesoporhyrin embedded in horseradish peroxidase. Biophys J 66: 577–582

Hewson WD, Hager LP (1979) Peroxidases, catalases and chloroperoxidases. In: Dolphin D (ed) The Porphyrins, vol 7. Academic Press, New York, pp 295–332

Koehler BE, Woehl JC (1995) Measuring internal electric fields with atomic resolution. J Chem Phys 102: 7773–7781

Köhler M, Gafert J, Friedrich J, Vanderkooi J, Laberge M (1996) Stark effect experiments in cytochrome-*c* type proteins: structural hierarchies. Biophys J 71: 77–85

Köhler M, Friedrich J, Balog E, Fidy J (1997) Symmetry breaking by the heme pocket in horseradish peroxidase as revealed by Stark spectroscopy. Chem Phys Lett 277: 417–422

Köhler M, Friedrich J, Fidy J (1998) Proteins in electric fields and pressure fields: basic aspects. Biochim Biophys Acta 1386: 255–288

Laberge M (1998) Intrinsic protein electric fields: basic non-covalent interactions and relationship to protein-induced Stark effects. Biochim Biophys Acta 1386: 305–330

Laberge M, Vanderkooi J, Koehler M, Friedrich J (1998). Probing structural heterogeneities in proteins by high resolution spectroscopy and electrostatic calculations. Biophys J 74: A250

Laberge M, Köhler M, Vanderkooi J, Friedrich J (1999) Sampling field heterogeneity at the heme of *c*-type cytochromes by spectral hole burning spectroscopy and electrostatic calculations. Biophys J 77: 3293–3304

Laird BB, Skinner JL (1989) Microscopic theory of reversible pressure broadening in hole-burning spectra of impurities in glasses. J Chem Phys 90: 3274–3281

Ortiz de Montellano PR, Ozaki S-I, Newmyer SL, Miller VP, Hartmann C (1995) Structural determinants of the catalytic activities of peroxidases. Biochem Soc Trans 23: 223–227

Sano S (1979) Reconstitution of hemeproteins. In: Dolphin D (ed) The porphyrins, vol 7, Academic Press, New York, pp 378–402

Schejter A, Lanir A, Epstein N (1976) Binding of donors to horseradish peroxidase: a spectroscopic study. Arch Biochem Biophys 174: 36–44

Schlichter J, Friedrich J, Herenyi L, Fidy J (2000) Protein dynamics at low temperatures. J Chem Phys 112: 3045–3050

Schonbaum GR (1973) New complexes of peroxidases with hydroxamic acids, hydrazides, and amides. J Biol Chem 248: 502–511

Smulevich G (1998) Understanding heme cavity structure of peroxidases: comparison of electronic absorption and resonance raman spectra with crystallographic results. Biospectroscopy 4: S3–S17

Teale FWJ (1959) Cleavage of the haem-protein link by acid methyl ethyl ketone. Biochim Biophys Acta 35: 543

Veitch NC (1995a) Substrate binding and catalysis in heme peroxidases. Curr Opin Chem Biol 2: 269–278

Veitch NC (1995b) Aromatic donor molecule binding sites of haem peroxidases. Biochem Soc Trans 23: 232–240

Voelker S, Macfarlane RM, Genack AZ, Trommsdorff HP, van der Waals JH (1977) Homogeneous linewidth of the $S_1 \rightarrow S_0$ transition of free-base porphyrin in an n-octane crystal as studied by photochemical hole burning. J Chem Phys 67: 1759–1765

Yonetani T, Yamamoto H, Erman JE, Leigh JS Jr, Reed GH (1972) Electromagnetic properties of hemoproteins. V. Optical and electron paramagnetic resonance characteristics of nitric oxide derivatives of metalloporphyrin-apohemoprotein complexes. J Biol Chem 247: 2447–2455

Zollfrank J, Friedrich J, Vanderkooi J, Fidy J (1991a) Conformational relaxation of a low-temperature protein as probed by photochemical hole burning. Biophys J 59: 305–312

Zollfrank J, Friedrich J, Fidy J, Vanderkooi J (1991b) Photochemical holes under pressure: compressibility and volume fluctuations of a protein. J Chem Phys 94: 8600–8603