HEME-LINKED IONIZATIONS OF MYELOPEROXIDASE DETECTED BY RAMAN DIFFERENCE SPECTROSCOPY

A Comparison with Plant and Yeast Peroxidases

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ABSTRACT The pH-dependence of the oxidation state marker line ν_4 of human leucocyte myeloperoxidase is determined in the absence of chloride using Raman difference spectroscopy (RDS). A transition in the frequency of ν_4 with pK of 4.2 ± 0.3 is found. The pK compares favorably with that previously determined by spectrophotometric titration and kinetic studies. The shift in ν_4 across the transition is -1.3 cm^{-1}. The shift in ν_4 and other Raman marker lines indicates enhanced π charge in the chlorin ring below the transition. The low frequencies of the oxidation state marker lines indicate that a structural change occurs near the chromophore, which results in the formation of a more π-charge donating protein environment for the chlorin ring at low pH. The Raman results are discussed in terms of a proposed catalytic control mechanism based on charge stabilization of the energy of ring charge–depleted ferryl intermediates of the reaction with peroxide. The myeloperoxidase findings are compared with similar RDS results for ferrous horseradish peroxidase and ferric cytochrome *c* peroxidase.

INTRODUCTION

Myeloperoxidase (MPO) is a multicomponent enzyme found in mammalian leucocytes that contributes to their bactericidal function (1–3). The active sites of the enzyme are two iron chlorins (see below) that are located in identical protein subunits. In the absence of halides MPO functions as a peroxidase that can catalyze the oxidation of a number of biological and nonbiological substrates using H_2O_2 as the oxidant (1). In the presence of chloride, the product is HOCl or Cl_2, both of which are potent bactericidal oxidizing agents (3, 4). The reaction mechanism involves formation of high oxidation state Fe-chlorin intermediates, as for other heme-containing peroxidases.

A number of investigations have sought to determine the catalytic mechanism and to elucidate the complex interrelationship between chloride concentration and pH effects on the catalytic rate (5–9). This work has focused on a heme-linked ionization that affects the catalytic rate when chloride is present (5). Spectrophotometric titration in the absence of Cl^- gives the pK of the transition in the range of 4.4–4.7 (8). This pH value is close to the physiologically relevant pH for MPO activity (5). Kinetic studies show that the rate of utilization of peroxide depends on both the concentration of Cl^-, if present, and pH (5, 7). To explain this interrelationship a reaction mechanism that involves ordered binding of the substrates H_2O_2 and Cl^- has been proposed (7–9). In this model, Cl^- binding requires protonation of the pK 4.4–4.7 group of the protein. Others suggest the existence of an inhibitory Cl^--binding site in addition to the substrate Cl^- site on the basis of kinetic results (5).

Recently, resonance Raman spectroscopy has proven useful as a structural probe in studies of peroxidase catalysis (10–15). Raman studies of MPO have determined the gross features of the chromophore in myeloperoxidase. The absence of the formyl Raman line and the presence of "extra" lines in the spectrum of MPO has led to identification of the chromophore as an iron chlorin (16–19). The effect of chloride binding on the Raman spectrum at pH 8 has also been reported, and pH-dependent photodegradation of MPO was noted in the absence of Cl^- at pH 4 (20).

Raman studies of heme-linked ionizations occurring in other peroxidases have centered primarily on the oxidation state marker line ν_4 and the iron–histidine stretching mode ν(Fe-his) (10–13). In every case the frequency of these two Raman lines has been found to undergo a transition with the characteristic pK of the particular peroxidase. In fact, for ferrous horseradish peroxidase (HRP), for which both Raman lines can be observed, the pH-dependent shifts in the two lines are linearly related and the frequencies show a transition with the relevant pK (11). The shifts are in the same direction for the two lines, and the shift for ν(Fe-his) is about nine times larger than for ν_4. This kind of relationship between ν_4 and ν(Fe-his) should be contrasted with that noted for R/T shifts in hemoglobins for which the two lines shift in opposite directions, and the shift in

ν(Fe-his) is only about five times as large as the shift in ν_4. The relationship between the shifts, $\Delta\nu$(Fe-his) and $\Delta\nu_4$, most likely are a result of a single structural change at the iron–protein linkage that affects both the Fe–histidine bond and, concomitantly, the porphyrin's π electron system. However, the different relationship between the two lines observed for hemoglobins and HRP isoenzymes points to a distinct mechanism of coupling between the Fe–histidine bond and the π electron system of protoporphyrin for these functionally distinct proteins. Therefore, it is of interest to determine the differences in structural changes in hemoglobins and peroxidases, and the relationship between these structural differences and differences in physiological function.

Arguments have been given for relating the electronic changes in the ring indicated by the shifts in ν_4 to catalytic activity of the peroxidases (11, 12), and this putative mechanism of catalytic control should be tested. In particular, comparisons among functionally distinct peroxidases are useful in answering these important questions; consequently, efforts are underway to investigate the relationship between Raman line shifts and catalysis for a variety of peroxidases.

With this goal in mind we have measured the pH-dependence of the Raman line assigned to ν_4 of native myeloperoxidase. (The Fe–histidine vibration has not been identified in the ferric protein.) The pH-dependence of ν_4 in the absence of Cl^- is reported here for comparison with similar pH-dependent peroxidase activity of HRP and cytochrome *c* peroxidase (C*c*P). As in the case of ferrous HRP isoenzymes and ferric C*c*P, we find ν_4 undergoes a transition with the pK (4.2 ± 0.3 for MPO) of a group known to control the rate of catalysis. Although without Cl^- present the pH-dependence of catalytic activity is weak for MPO, the changes in the absorption spectrum of MPO are similar to those observed when Cl^- is present and the effect of pH on catalytic activity is large. The significance of the pH-dependence of ν_4 of MPO is discussed in terms of a proposed catalytic mechanism.

MATERIALS AND METHODS

Myeloperoxidase was isolated and purified by the method of Mansson-Rahemtulla et al. (Mansson-Rahemtulla, B., D. C. Baldome, K. M. Pruitt, and F. Rahemtulla, manuscript submitted for publication) from neutrophils obtained from freshly leucopheresed blood or were gifts from Kenneth Pruitt of the University of Alabama at Birmingham or Robert Clark of the University of Iowa. The desired pH of the MPO samples in 0.2 M phosphate buffer was established by dialyzing against the 0.2 M phosphate buffer at the required pH. The buffers were prepared by mixing mono-, di-, and tri-phosphate stock solutions. The appropriate amount of phosphoric acid was added to obtain the lowest pH solutions. The pH was measured after the Raman spectrum using a pH meter equipped with a long, thin electrode (model Φ70; Beckman Instruments, Inc., Palo Alto, CA). The concentration of the MPO solution was typically 1×10^{-4} M based on an extinction coefficient of 6.7×10^4 $M^{-1}cm^{-1}$.

Raman difference spectra were taken using a difference spectrometer described previously (22–24). The Raman difference spectroscopic technique allows frequency shifts between corresponding lines from two samples to be measured to an accuracy of ~ 1/100th of the line width or ~0.1 cm^{-1} in the case of MPO (24). Raman spectra were obtained using 90° scattering with the 413.1-nm line of a krypton ion laser (model 3000K; Coherent, Inc., Palo Alto, CA). Typical power levels used were <200 mW with a slightly defocused scattering column. The spectra were obtained at room temperature in a rapidly rotating (100 Hz) two-compartment cell. Under these conditions no local heating occurs, even at 200 mW. All data were obtained in no more than 1 h except in studies of MPO photodecomposition. Each scan of the 100-cm^{-1} region took 5 min. The spectral resolution is 4 cm^{-1}.

Myeloperoxidase at pH 7.1 ± 0.1 was used as a reference for comparison with the protein at other pH values in the range from 3.0 to 9.5. The reference pH was chosen because photodecomposition is less of a problem at high pH and because preliminary RDS experiments had shown that ν_4 does not vary with pH in the vicinity of neutral pH (Fig. 3).

Photodecomposition is a serious problem for MPO partly because it occurs on the time scale required for typical Raman spectra (>2 h) and because the photodecomposition product is also resonance enhanced. Photodecomposition is not a result of sample heating. Resonance enhancement of the photoproduct results in interfering lines in the spectrum of MPO. Flow techniques, in this case, do not provide a viable method for avoiding the problem because of the small quantity of MPO available to us. Instead, we made relatively short runs (<1 h) to obtain small regions of the Raman spectrum, in particular, the region about the oxidation state (25–27) marker line ν_4. We also used relatively concentrated MPO solutions with the maximum volume (250 μl) possible with our rotating two-compartment cell.

Finally, as a further means of overcoming the interference from the photoproduct, we used a novel method of analysis to determine the differences in frequency of ν_4. Fig. 1 shows a typical comparison of simultaneously obtained Raman spectra for MPO at different pH values (pH 4 and pH 7) and serves to demonstrate the method used in determining the frequency shift $\Delta\nu_4$. The top (pH 3.9) and bottom (pH 7.0) spectra in Fig. 1 *a* shows the Raman spectra obtained within 1 h. Each spectrum is decomposed into component Lorentzian lines using a nonlinear least-squares program in which (*a*) the position of the maximum, (*b*) the peak intensity, and (*c*) the line width of each of five lines serve as the parameters that are varied to obtain the least squares fit shown. The background level is also allowed to vary. The number of data points in the 100-cm^{-1} region that are used for the Lorentzian decomposition is 250. The fits obtained are reasonably good; however, better fits were obtained in the region between the two main peaks when an additional line is included on the high frequency side of ν_4 between the two peaks. Although better fits were obtained, confidence limits on the peak positions were much greater for these fits and could not be used. Thus, we sacrificed using a better fitting procedure for a less precise but unique measure of the pH-dependent changes in the spectrum. Approximate Voigt lineshapes were also tried, but the Gaussian contribution to the lines was found to be small (<5%) and the root-mean-square error for the fits were nearly the same as for fits using Lorentzian lineshapes.

Frequency shifts in ν_4 are determined from the Lorentzian decomposition by subtracting the center positions of the line corresponding to ν_4 in each spectrum (Fig. 1 *a*, *top* and *bottom*) given by the best fit. The error in the position of ν_4 is typically ±0.1 cm^{-1} for each spectrum, so an error of ±0.2 cm^{-1} is reported for $\Delta\nu_4$. The shift is -0.67 ± 0.20 cm^{-1} for the pH 4 minus pH 7 data of Fig. 1 *a*.

The Lorentzian decomposition method was found to give a more reliable (and less pathological) determination of $\Delta\nu_4$ than the Raman-difference-spectrum method usually used (23, 24). As a test of the possible effect of photodegradation on our shift determinations, the samples of Fig. 1 *a* were further exposed to laser radiation for another hour, and then the spectra were again taken (requiring an additional half hour in the beam). The spectra obtained are shown in Fig. 1 *b* along with the results of the Lorentzian decomposition. Significant photodecomposition is observed by this time, and the spectrum of the photoproduct is most evident in the "filling-in" between the two strongest lines. This filling-in is

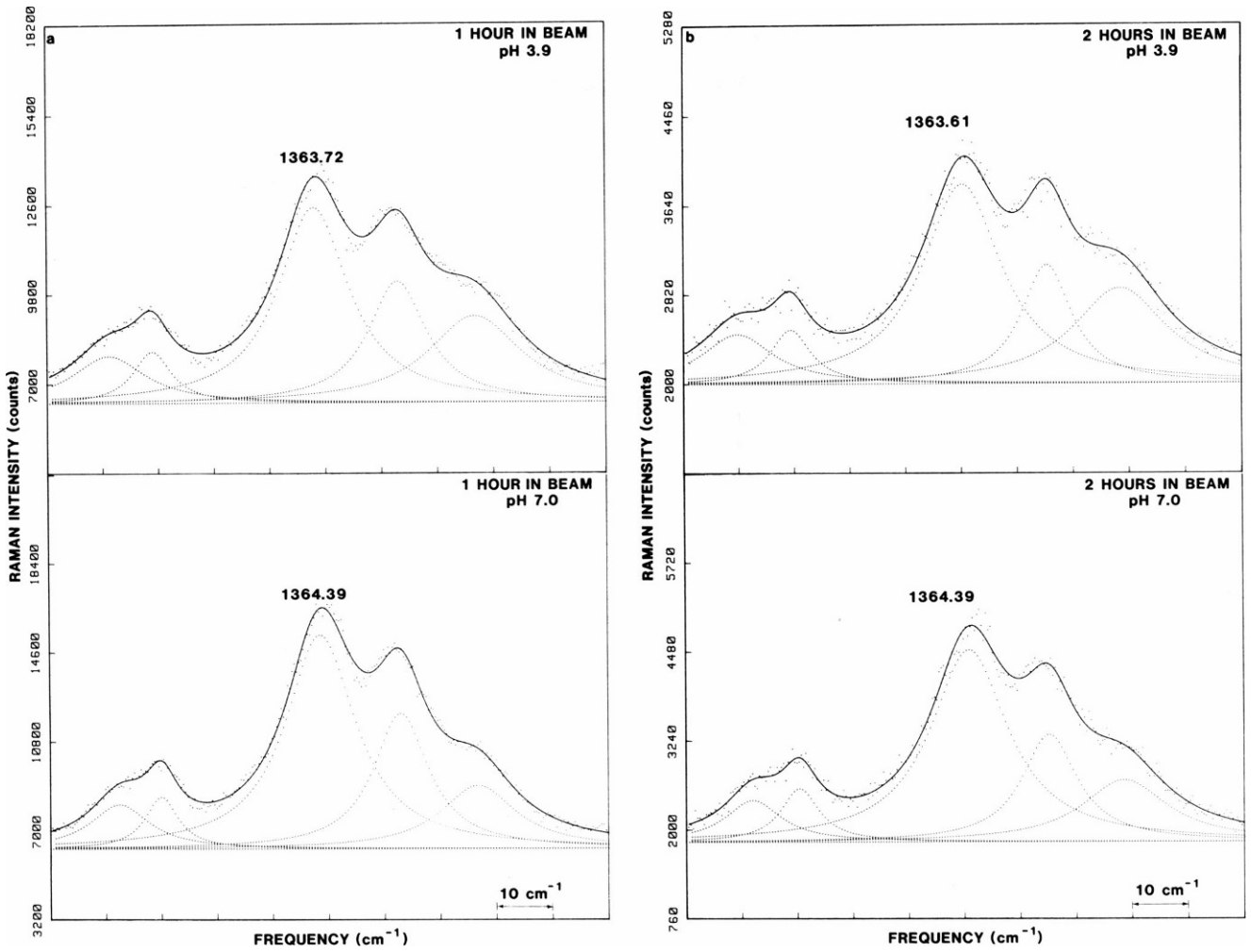

FIGURE 1 Lorentzian decomposition of simultaneously obtained RDS spectra in the neighborhood of the oxidation state marker line ν_4 of myeloperoxidase at pH 3.9 (*top*) and pH 7.0 (*bottom*) after 1 h in laser beam (*a*) and after over 2 h in the beam (*b*).

best illustrated in Fig. 2, which compares the spectra obtained in 1 h and after 3 h and shows the difference between the two. The frequency shift obtained from the Lorentzian decomposition of the data in Fig. 1 *b* is -0.78 ± 0.31 cm^{-1}. The difference between the two determinations (0.11 cm^{-1}) is not significant. In fact, it most likely reflects the difference in fits due to the higher noise level (resulting from a shorter data collection time) in the spectra of the more photodegraded MPO. Therefore, we are confident that the reported shifts result from pH differences and not the differences in degree of photodecomposition of MPO. This conclusion is supported by the fact that the large pH shifts that we measured are clearly evident on the first 4-min scan of the Raman spectrum during which there is no evidence of photodecomposition.

RESULTS AND DISCUSSION

Photodecomposition is a serious problem in Raman studies of myeloperoxidase. Our Raman data are obtained within a time interval for which photodegradation of MPO is minimized as described in the previous section. The initial photodecomposition of MPO results in the appearance of a new line at 1,371 cm^{-1} between ν_4 at 1,364 cm^{-1} and the line at 1,376 cm^{-1}. Both lines of native MPO lose intensity (although the 1,376-cm^{-1} line is most affected) as the new line grows in between their maxima (Fig. 2). After long irradiation times at higher power only one peak with a high frequency shoulder is observed in the region. MPO is photolabile at high as well as low pH where photodecomposition was previously recognized (20). No mention of MPO photochemistry was made in other Raman studies (16, 17). Photodecomposition is likely minimal in the work of Babcock et al. (17) because of the large sample volume used and the presence of the resolved line at 1,378 cm^{-1} in their spectrum of ferric MPO.

Ikeda-Saito et al. (20) noted that near physiological concentrations of Cl^- protect MPO from photodegradation to some degree (20). In addition, large spectral differences were observed in the region about ν_4 in the presence and absence of chloride (0.2 M) at pH 8. However, the reported spectrum of MPO in the absence of Cl^- shows evidence of photodegradation. We have compared MPO at pH 8 in 0.2 M phosphate buffer and in 0.1 M phosphate-0.1 M Cl^-, and using the method described in the previous section we observe no significant (<0.25

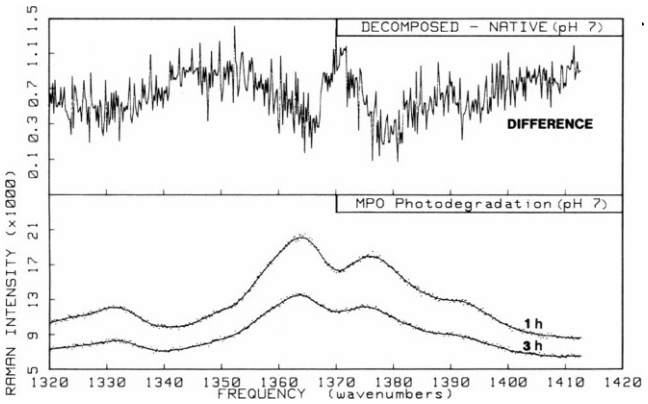

FIGURE 2 Raman spectra of myeloperoxidase in the region of the oxidation state marker line ν_4 showing the effect of early photodegradation on the Raman difference spectrum.

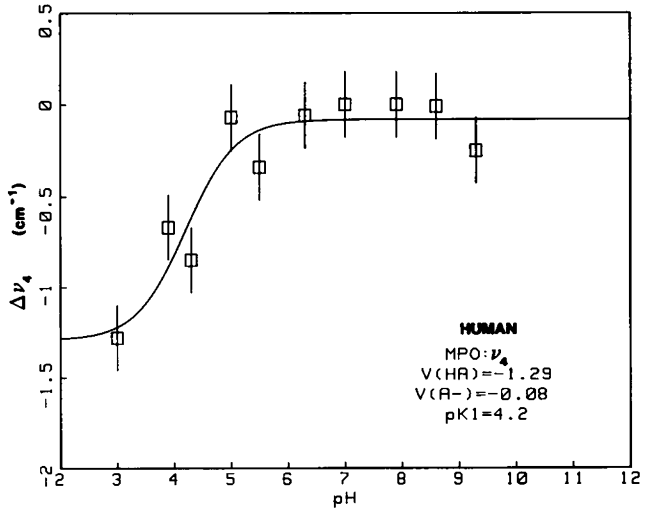

FIGURE 3 The pH-dependence of the frequency shift of the oxidation state marker line ν_4 relative to the frequency at pH 7.

cm^{-1}) differences in the region 1,320–1,420 cm^{-1}. In particular, the high frequency line at 1,374 cm^{-1} remains resolved, and, in fact, the difference spectrum remains flat throughout the region. Therefore, it appears that the reported Cl^- differences at pH 8 result from undetected photodecomposition of MPO, not from a large chloride binding effect.

The oxidation state marker line of heme protein (26) and metalloporphyrin (26, 27) Raman spectra has also been identified in the spectra of model iron chlorins (25). Because of their lower symmetry relative to the iron porphyrins, the spectra of the Fe chlorins contain many additional lines. In particular, the region about ν_4 of the Fe chlorins exhibits several strong lines. The ν_4 region of myeloperoxidase shown in Fig. 1 also exhibits several lines not observed in porphyrin analogues. For five-coordinate model ferric chlorins, ν_4 is at 1,367 cm^{-1}, and for six-coordinate ferric chlorins, ν_4 is at 1,370 cm^{-1}. The neighboring high frequency line near 1,390 cm^{-1} has been assigned, by analogy to the porphyrin modes, to ν_{12}; the low frequency companion near 1,342 cm^{-1} is assigned to ν_{41} (25).

For myeloperoxidase, ν_4 occurs at a lower frequency (at 1,364 cm^{-1}) than for the model chlorins. This probably indicates a slightly more π donating fifth ligand than the Cl^- of the model Fe chlorin. Imidazole is one possible ligand.

The oxidation state and core size marker lines (26, 28), ν_3 and ν_{10}, appear in Raman spectra of the model ferric chlorins at 1,476 cm^{-1} and 1,608 cm^{-1} for six-coordinate models and at 1,489 cm^{-1} and 1,631 cm^{-1} for five-coordinate models, respectively (25). The lines appear at 1,481 cm^{-1} (ν_3) and 1,614 cm^{-1} (ν_{10}) for myeloperoxidase at pH 7 (see Fig. 4). The frequencies are closer to five-coordinate values than to six-coordinate ones, as has been noted previously (16–18).

The pH-dependence of ν_4 is shown in Fig. 3. The oxidation state marker line undergoes a transition with pK of 4.2 ± 0.3 assuming a single proton is titrated. The shift in ν_4 of 1.2 ± 0.2 cm^{-1} is quite large when compared, for example, with the R/T shift in ν_4 for methemoglobins of 0.6–0.8 cm^{-1} and the pH-dependent shift in ferric*Cc*P of 0.8 cm^{-1} (10, 29). Slightly larger shifts are noted upon a change in axial ligand and spin state at high pH for ferroHRP and C*c*P.

The usual interpretation of shifts in ν_4 is in terms of a change in charge density delocalized in the porphyrin ring's π electron system. For model iron(II) porphyrins, a change in π charge density is accompanied by a shift in ν_4 and by similar sized shifts in the marker lines sensitive to core size and oxidation state (30). These latter Raman lines include ν_{10} and ν_3. As can be seen in Fig. 4, ν_3 and ν_{10} of MPO shift to low frequency at pH 3 relative to pH 7. Because all of these lines exhibit similar shifts to low frequency at low pH, we conclude that the pattern of shifts in this group of marker lines is consistent with a change in π charge density in the chlorin ring.

Based on this interpretation of the marker line shifts, we conclude that the low frequencies of ν_4, ν_3, and ν_{10} represent an increase in the π charge resulting from a relatively more charge donating (or less charge accepting) protein environment at acid pH. In contrast with MPO, the acid transition of C*c*P and ferroHRP results in the protein donating less π

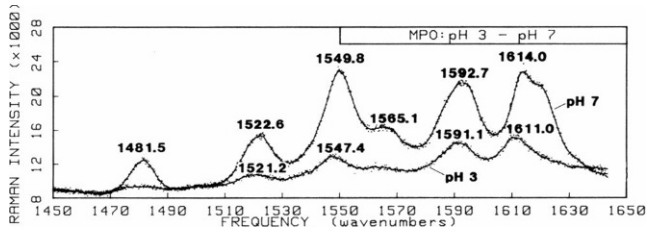

FIGURE 4 Raman spectra of myeloperoxidase at pH 3 and pH 7 obtained within 1 h showing the large (>1 cm^{-1}) shifts in the high frequency core size and oxidation state marker lines.

charge to the ring (since ν_4 shifts to high frequency at low pH). We speculate that the different response to pH for these peroxidases may reflect their functional differences, e. g., the inability of HRP and CcP to oxidize chloride.

Previously, Shelnutt and co-workers (10, 11) have suggested a mechanism of pH-dependent control of catalytic activity for the non-chloride-oxidizing peroxidases based on π charge in the porphyrin ring. Increased ring charge would presumably lower the energy of the transition state of the reaction, which involves highly charge-depleted ferryl intermediates. At pH values for which high ring π charge is inferred from the low frequency for ν_4, the energy of high oxidation state intermediates (compounds I and II) is stabilized relative to pHs for which ν_4 indicates low ring charge. In this way π charge stabilization of compounds I and II provides a viable mechanism that may contribute to pH-dependent control of peroxidase activity. Indeed, cytochrome *c* peroxidase and ferrous horseradish peroxidase exhibit high ring charge (low ν_4) (10, 11) at pH values for which maximal catalytic activity is observed (31–35).

For myeloperoxidase, on the other hand, only a small change in catalytic activity is noted for oxidation of a substrate such as tetramethylbenzidine in the pH range from 4 to 7. On the basis of the decrease in ν_4 of MPO at low pH, one would expect an increase in catalytic activity. A slight increase is observed at low pH (5). Nevertheless, by comparison with other peroxidases the shift of 1.2 cm^{-1} for MPO would suggest a greater effect on activity.

The weak dependence of peroxidase activity on pH in the absence of Cl^- is somewhat surprising given that the change in the absorption spectrum with pH is similar to the pH-dependent spectral changes in MPO with Cl^- present and large differences in activity then occur. The interrelationship between Cl^- and protonation of the pH 4.5 group has not been addressed in the present study; therefore, the role of porphyrin ring charge as a control mechanism has yet to be determined for MPO. Nevertheless, it is clear that for all peroxidases investigated so far π ring charge is controlled by pH and a reasonable mechanism by which π charge can control activity exists. Factors other than π ring charge, such as direct interaction of the ionizable group with the substrates Cl^- and H_2O_2, will also influence catalytic rates. These other factors must also be included along with heme electronic structure considerations in determining the observed reaction rate. It has been suggested for MPO that Cl^- binding to a protonated pK 4.5 group influences H_2O_2 binding, causing inhibition of Cl^- oxidation (5). Protonation in the absence of Cl^- could still affect H_2O_2 or substrate binding directly and, thereby, compensate for the enhanced rate predicted on the basis of π charge effects. Further comparative RDS studies of heme-linked ionizations may help to sort out the relative contributions of these pH-dependent structural changes to the control of catalytic rates.

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