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Optical spectrum of myeloperoxidase
Origin of the red shift

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The optical spectrum of reduced myeloperoxidase (EC 1.11.1.7) displays an unusual red shift of the Soret band which is at 472 nm and the a-band which is at 636 nm. The spectral properties of myeloperoxidase can be modified by means of acid treatment. Upon short exposure to acid (pH 1.7) the red-shifted optical absorption spectrum of the reduced enzyme ($\lambda_{\text{max}}$ at 472 nm) was blue-shifted ($\lambda_{\text{max}}$ at 448 nm) but the spectrum of the reduced state could be restored by increasing the pH. By contrast, the resonance Raman spectra of both the oxidized and reduced enzyme are essentially the same at both pH 1.7 and pH 7.0. This shows that the optical spectrum and the resonance Raman spectrum are not directly correlated, which we interpret to indicate that the reversible effects of lower pH primarily affect the excited-state energy levels of the macrocycle. The EPR spectrum of the oxidized enzyme showed a reversible conversion from a high-spin rhombic spectrum ($g_x = 6.7$, $g_y = 5.2$) at neutral pH into a more axial high-spin spectrum ($g_x = g_y = 5.8$) at low pH.

Upon prolonged exposure to acid (20 min) optical absorbance spectra, EPR spectra, resonance Raman spectra and the chlorinating activity were irreversibly affected. We propose that a negatively charged protonatable residue in the proximity of a pyrrole nucleus of the haem group is present that imposes the red shift in the optical absorption spectrum. This is consistent with the available X-ray structure data.

Myeloperoxidase is present in large amounts in polymorphonuclear neutrophils and plays an important role in the antimicrobial activity. The enzyme catalyzes the $\text{H}_2\text{O}_2$-dependent peroxidation of chloride to hypochlorous acid, which is a bactericidal agent [1, 2]. Myeloperoxidase has also been shown to react very rapidly with peroxynitrite and a new role for the enzyme as a scavenger for peroxynitrite has been proposed [3].

The optical absorption spectrum of the reduced enzyme is unique because the Soret band (472 nm) and the a-band (636 nm) are red-shifted compared with other haem proteins [4]. The chemical nature of the prosthetic group of myeloperoxidase, which is a haem group, has, however, not been identified, partly due to the fact that the haem group is not readily extractable. This is possibly the result of a covalent linkage between the prosthetic group and the protein [5–7]. The crystal structure has been determined at 0.3-nm resolution [8], however, the exact nature of the prosthetic group could not be established from the current X-ray data. It has been suggested that the chromophore is a porphyrin with electron-withdrawing substituents because pyridine haemochrome spectra and the spectra of the enzyme treated with SDS or acid have a strong similarity with that of the haem a spectra of the corresponding form of cytochrome c oxidase [9–13].

Carbonyl reagents have been shown to react with myeloperoxidase, suggesting that a formyl group is present on the porphyrin similar to haem a [5, 9–13]. However, as opposed to these observations, the stretching vibration of a formyl substituent, in conjunction with the macrocyclic ring, has not been observed by resonance Raman spectroscopy. Apart from the evidence suggesting a haem-a-like prosthetic group, magnetic circular dichroism (MCD) and resonance Raman studies indicate that the chromophore is a chlorin-type haem, in which a pyrrole ring of the porphyrin is saturated [14–20]. In addition, it has been suggested that the chlorin may bear a peripheral formyl substituent not in conjunction with the macrocycle [18]. From both resonance Raman and NMR studies, it has been suggested that two vinyl groups are present on the haem periphery [18, 21]. Recently a 1H-NMR and NOE study of myeloperoxidase [22] and spectroscopic studies of photochemically modified enzyme [21] have been reported, both of which suggest that the prosthetic groups of myeloperoxidase and lactoperoxidase are similar, suggesting that myeloperoxidase contains a porphyrin. From MCD studies on haem-reconstituted myoglobin, evidence has been obtained that the prosthetic group of myeloperoxidase is a formyl-substituted porphyrin [23]. It is, however, not possible presently with the available MCD, resonance Raman, 1H-NMR and optical data on myeloperoxidase to give a detailed structure of the chromophore and active site that can explain all the spectral features, in particular the red-shifted optical spectra of the reduced enzyme. In view of these data it has been suggested that the protein part of the enzyme greatly affects the electronic structure of the haem group [4].
Recent X-ray crystallographic data [8] show that carboxylic residues are present in close proximity to the haem macrocycle. These negatively charged residues may provide the major interaction of the protein matrix with the chromophore to cause the red shift in the optical spectrum. Protonation of a negatively charged residue close to the haem will eliminate the effect of the negative charge on the electronic properties of the prosthetic group.

Indeed, when myeloperoxidase is exposed to acid, the enzyme is converted into a form of the enzyme in which the optical spectrum of the reduced state is blue-shifted and this may be related to the protonation of these groups. In the present study, we show that the effect of acid on the various spectral properties is reversible. Furthermore, the correlation between the unique spectral properties of the enzyme and the ability to peroxidize chloride is investigated.

**MATERIALS AND METHODS**

**Enzyme preparation**

Human myeloperoxidase was prepared from leukocytes according to standard procedures [24]. The enzyme concentration was measured spectrophotometrically using a molar (with respect to haem) absorption coefficient of 89 mM$^{-1}$ cm$^{-1}$ at 428 nm [25]. The spectral ratio $A_{428\text{nm}}/A_{280\text{nm}}$ of the enzyme preparations was at least 0.75.

**Activity measurements**

Myeloperoxidase (2µM) was incubated in 19 mM HCl and, at intervals, 45-µl aliquots of the acid-exposed enzyme solution were added to an optical cuvet containing 50 µM monochlorodimedone and 126 mM H$_2$O$_2$ in 2.5 ml 19 mM HCl and the conversion of monochlorodimedone (1,1-dimethyl-4-chloro-3,5-cyclohexadione, $\epsilon = 20.2$ mM$^{-1}$ cm$^{-1}$ at 290 nm) into dichlorodimedone (ε = 0.2 mM$^{-1}$ cm$^{-1}$ at 290 nm) monitored at 290 nm was used for measuring the chlorinating activity [26]. At the same time intervals, another 100-µl aliquot from the same acid-exposed enzyme solution was mixed with 100 µl 100 mM sodium phosphate pH 7.2 and was allowed to stand for 30 min. After that, 90-µl aliquots (to compensate for the 1:1 dilution) were added to an optical cuvet containing 50 µM monochlorodimedone and 126 mM H$_2$O$_2$ in 2.5 ml 19 mM HCl and the absorbance decrease at 290 nm was followed to measure the chlorinating activity. The activity measurements were performed on a Cary-17 spectrophotometer.

**Optical measurements**

17 mM HCl was added to myeloperoxidase and, at selected times, samples were taken from this incubation and transferred to an optical cuvet containing 17 mM HCl. Within 15 s the optical absorption spectra of the oxidized and the reduced enzyme were recorded. Samples of the enzyme incubated in acid were mixed with the same volume of 100 mM sodium phosphate pH 7.2 directly after which the optical spectra of both the oxidized and reduced enzyme were recorded. Dithionite was used for reduction and the optical spectra were recorded on a Hewlett Packard 8452 A diode array spectrophotometer.

**Electron spin resonance experiments**

Myeloperoxidase (15 µM) was exposed to 19 mM HCl for 75 s, immediately after which the samples were frozen in isopentane and the EPR spectra were recorded. To identical incubations, a small amount of 1 M sodium phosphate was added to adjust the pH to 7.4 after 75 s and subsequently the EPR spectra were recorded. The EPR spectra were recorded at 8 K using helium flow on a Varian E-9 spectrometer.

**Resonance Raman experiments**

The resonance Raman spectra were recorded using a diode array detection system (OMA-III, PAR, EG & G) with a triple monochromator (Spex 1877) and the samples were spun during irradiation except for the samples of reduced enzyme. A Coherent model 90 K krypton ion laser was used for the Raman excitation at 431.3 nm and a Liconix NaCd laser was used for the Raman excitation at 446.1 nm. The optical spectra were checked before and after the resonance Raman experiments. The concentration of acid (HCl) used for the resonance Raman experiments was 17 mM.

**RESULTS**

The optical absorption spectrum of the native oxidized enzyme shows a Soret band at 428 nm and a band at 570 nm. Upon reduction of the enzyme the Soret band shows an unusual red-shifted position with $\lambda_{\text{max}}$ at 472 nm and similarly the $a$-band is observed at 636 nm (not shown). When the native oxidized enzyme was incubated in acid for a short period (50 s) the spectrum remained unchanged and was nearly identical to that of the native enzyme (not shown). However, the spectrum of myeloperoxidase that had been incubated in acid for a short period (50 s) after which the enzyme was reduced, changed (Fig. 1) and was blue-shifted as compared with the spectrum of the ferrous native enzyme. The Soret band shifted from 472 nm to 448 nm and the $a$-band shifted from 636 nm to 595 nm. The acid-induced conversion of the optical absorption spectra was reversible since, upon diluting the acidic oxidized enzyme solution in sodium phosphate pH 7.2, the original spectrum was restored and the sample that was reduced after this treatment displayed a Soret band at 472 nm and an $a$-band at 636 nm, which are identical to those of the native enzyme (Fig. 1).

The reversibility of the acid treatment was critically dependent on the HCl concentration. A concentration of 15-20 mM HCl was required, at higher HCl concentrations the conversion of the enzyme into the form of which the optical absorption spectrum was irreversibly changed was rapid. This implies that the enzymic species for which the optical spectrum is reversibly blue-shifted existed for only a short time at these HCl concentrations. On the other hand, at lower HCl concentrations, more time was required to fully convert the reduced enzyme with its Soret band at 472 nm into the species with a Soret band at 448 nm. At 10 mM HCl, it required about 8 min before conversion was complete and the optical spectrum showed a single peak at 448 nm.

The EPR spectrum of myeloperoxidase displays a rhombic high-spin signal (Fig. 2, spectrum A) with $g_x = 6.7$ and $g_y = 5.2$. The effect of acid on the EPR spectrum was also studied. Myeloperoxidase was incubated in acid for a short period, after which the sample was rapidly frozen in isopentane. The EPR spectrum showed a more axial-type high-spin signal ($g_x = g_y = 5.8$) as compared with that of the native enzyme (Fig. 2, spectrum B). On this time scale the effect of acid was reversible. Fig. 2, spectrum C, shows that the EPR spectrum of myeloperoxidase that was incubated in acid for
Fig. 1. Optical absorbance spectra of reduced myeloperoxidase. (- - - - -) Myeloperoxidase was incubated in 17 mM HCl for 50 s after which the enzyme was reduced and the optical spectrum of the enzyme in the Raman cell was recorded using a diode array spectrophotometer (this sample was used to record the resonance Raman spectrum of Fig. 3b, trace B). (——–) Myeloperoxidase was incubated in 17 mM HCl for a short period (50 s) after which the pH was increased to pH 7.0 and the enzyme was reduced (this sample was also used to record the resonance Raman spectrum of Fig. 3b, trace C).

a short period, after which the pH was increased to neutral pH, displays again a high-spin signal with a slightly higher rhombic symmetry ($g_x = 6.9$ and $g_y = 5.1$) than the native enzyme.

The resonance Raman spectra of myeloperoxidase are rather complex compared with the spectra of conventional haem proteins. This has been explained by symmetry reduction effects and the vibrational data are consistent with an iron chlorin as the prosthetic group [14-18]. It has, however, been shown [27] that denaturation with guanidine • HCl results in resonance Raman spectra of the oxidized enzyme that are less complicated with essentially a single line in the oxidation-state marker region ($v_a$) at 1379 cm$^{-1}$, characteristic of a normal porphyrin. In the spectrum of the denatured reduced enzyme, which displays a band at 448 nm in the optical spectrum, the oxidation-state marker was shifted to lower wavenumber (1363 cm$^{-1}$).

The effects of acid on the resonance Raman spectra were studied. Fig. 3a shows the resonance Raman spectrum of the oxidized native enzyme at neutral pH (trace A) and of that which was exposed to acid for a short period (trace B). This treatment only slightly affected the spectrum. Neutralization yielded a spectrum (Fig. 3a, trace C) which was nearly identical to that of the native enzyme. This shows that during the treatment the oxidation-state marker remained unchanged, i.e. that multiple lines, characteristic of symmetry lowering effects were observed.

Fig. 2. EPR spectra of myeloperoxidase. Spectrum A, 15 μM myeloperoxidase in 100 mM sodium phosphate pH 7.4. Spectrum B, 15 μM myeloperoxidase incubated in 19 mM HCl for 75 s. Spectrum C, 15 μM myeloperoxidase incubated in 19 mM HCl for 75 s after which the pH was increased to pH 7.4. Conditions used during the recording of the spectra were: temperature, 8 K; frequency, 9261 MHz; modulation amplitude, 1 mT; microwave power incident to the cavity, 32 mW; gain, 4×10$^3$.

Fig. 3b, trace A, shows the resonance Raman spectrum of the reduced native enzyme. When the enzyme was incubated in acid for a short period and reduced the optical absorbance spectrum displayed a Soret band at 448 nm, but the corresponding resonance Raman spectrum (Fig. 3b, trace B) of this species was only slightly affected. Neutralization and reduction of the species that was briefly incubated in acid resulted in a resonance Raman spectrum (Fig. 3b, trace C) that is indistinguishable from that of the native reduced enzyme. The optical absorption spectrum of the neutralized enzyme solution after reduction displayed again a Soret band at 472 nm. These experiments demonstrate that the effects of acid on this short time scale on the resonance Raman spectra are negligible and that the changes in the optical spectrum do not result in changes in the resonance Raman spectra.

Studies on photochemically modified myeloperoxidase, for which the absorption spectrum of the reduced enzyme was irreversibly blue-shifted ($λ_{max}$ at 446 nm), indicated that the unusual red-shifted optical spectrum is not a prerequisite for the ability to peroxidize chloride [21]. In contrast, the guanidine • HCl denaturation/renaturation experiments [27] suggested a correlation between the unique spectral properties and the chlorinating activity. However, this correlation may have been incidental. Therefore, the effect of exposure of myeloperoxidase to acid was used to study the correlation between the spectral properties and the chlorinating activity. The chlorinating activity of the native enzyme at pH 5 (100 mM Cl$^-$, 100 μM H$_2$O$_2$) was identical to the chlorinating activity under the same conditions of the enzyme that was incubated in acid for a short period (90–120 s). This
Fig. 3. Effects of a short incubation of myeloperoxidase in acid on its resonance Raman spectra. (a) The resonance Raman spectra were obtained using 20-mW laser power at 413.1 nm and the resonance Raman data were accumulated during 30 s. (A) Native (oxidized) myeloperoxidase (30 pM) in 40 mM sodium phosphate pH 7.0. (B) Myeloperoxidase (40 pM) incubated in acid for 50 s. (C) Myeloperoxidase (30 pM) incubated in acid for 50 s after which the solution was neutralized (pH 7.0). (b) The resonance Raman spectra were obtained using 20-mW laser power at 441.6 nm and the resonance Raman data were accumulated during 300 s in A, 30 s in B and 180 s in C, respectively. (A) Native reduced myeloperoxidase (40 pM) in 40 mM sodium phosphate pH 7.0. (B) Myeloperoxidase (40 pM) was incubated in acid for 50 s after which the enzyme was reduced. The optical spectrum was checked before the resonance Raman spectrum was accumulated (Fig. 1). (C) Myeloperoxidase (30 pM) was incubated in acid for 50 s after which the pH was increased to pH 7.0 and the enzyme was reduced.

implies that short exposure to acid (19 mM HCl) has no effect on the chlorinating activity. To study the correlation of the spectral changes induced by incubation in acid and the chlorinating activity, this activity was measured in 19 mM HCl as function of time. When myeloperoxidase is exposed to 19 mM HCl the chlorinating activity remains unaffected in the first 90 s, after which there is a gradual decrease in chlorinating activity. In these experiments a high concentration of H₂O₂ had to be used, since at low pH the Kₘ for H₂O₂ is high, and chloride strongly inhibits the chlorination reaction at low pH. Direct oxidation of monochlorodimedone by H₂O₂ and myeloperoxidase at low pH that has been reported in [2] was not observed. It is possible that the effect of the prolonged acid incubation on the chlorinating activity is reversible. If this is the case, increasing the pH should restore the chlorinating activity. Therefore, myeloperoxidase was incubated in 19 mM HCl for a certain period and the pH was increased to pH 7.2 and, subsequently, the chlorinating activity was measured in 19 mM HCl. Within the first 90 s of incubation a chlorinating activity was observed that was identical to that of the enzyme that was only exposed to acid once. Thus, short exposure to acid has no effect on the chlorinating activity. However, after this period, a gradual decrease in chlorinating activity was observed that cannot be restored by neutralization of the enzyme solution and apparently the enzyme becomes inactive. That irreversible changes occur is also evident from the optical characteristics of the enzyme.

Upon prolonged incubation in acid (20 min) the spectrum of oxidized myeloperoxidase changed: the Soret band shifted
from 430 nm to 412 nm, the band at 570 nm shifted to 554 nm and isosbestic points were observed at 422 nm and around 550 nm (not shown). Neutralization did not restore the spectrum of native myeloperoxidase, and bands were observed at 430 nm and around 590 nm. The Soret band of the reduced enzyme after prolonged incubation in acid was observed at 442 nm. The effect of acid could not be reversed and after neutralization the Soret band of the reduced enzyme was found at 448 nm.

Similarly the EPR spectrum of the enzyme that was incubated in acid for a longer period (20 min) was axial ($g_x = g_y = 5.9$) and similar to that after a short incubation, but after neutralization the EPR spectrum displayed a very broad signal, which we were unable to interpret (not shown).

Irreversible changes are also observed in the resonance Raman spectrum of the oxidized enzyme upon prolonged exposure to acid (Fig. 4a, trace A). In the oxidation-state marker region, a more symmetrical band was observed at 1379 cm$^{-1}$, After neutralization the oxidation-state marker band remained at 1379 cm$^{-1}$ (Fig. 4a, trace B), the resonance Raman spectrum remained symmetrical and a new mode is now observed at 1642 cm$^{-1}$. This spectrum is different from that of the native enzyme.

The resonance Raman spectrum of the reduced enzyme also changed when myeloperoxidase was incubated in acid for a longer period. This is illustrated in Fig. 4b, traces A – C, where resonance Raman spectra are shown at various time intervals during the incubation in acid. From this figure it is clear that the Raman spectrum becomes less complicated during the incubation, the multiple lines in the oxidation-state marker region collapse to a single intense line and there is a shift from 1353 cm$^{-1}$ to 1360 cm$^{-1}$. Again, these changes upon prolonged incubation in acid are not reversible. When myeloperoxidase was incubated in acid for 20 min and the solution was neutralized, the resonance Raman spectrum of the reduced enzyme was different to that of the native enzyme. In the oxidation-state marker region a single symmetrical intense band was observed at 1360 cm$^{-1}$ (Fig. 4b, trace D).

**DISCUSSION**

Short incubation of the enzyme in acid affects the optical, resonance Raman, EPR and enzymic properties of myeloperoxidase in a reversible process, since increasing the pH led to restoration of the properties of the native enzyme. Upon prolonged exposure to acid, the effects of low pH are no longer reversible and the properties of the native enzyme could not be restored by increasing the pH. Fig. 5 summarizes the properties of the various species which have been observed in this study and their interconversion.

Upon short incubation in acid the spectrum of the oxidized enzyme was only slightly affected, which is due to formation of the spectroscopically detectable myeloperoxidase-chloride complex (species O$_2$ in Fig. 5) at low pH with a Soret band maximum at 340 nm [28]. After increasing the pH (species O$_2$ in Fig. 5), the spectrum of the native enzyme was recovered. The optical spectrum of the reduced enzyme ($\lambda_{max}$ at 472 nm) was converted into a blue-shifted optical spectrum ($\lambda_{max}$ at 448 nm) by short exposure to acid. The spectrum of this species (R, in Fig. 5) resembles the spectrum observed after denaturation with 5 M guanidine·HCl [27]. It also resembles that of the acid-modified form of myeloperoxidase, referred to as ‘acid myeloperoxidase’ [10].

The native spectrum could be fully recovered by increasing the pH after the incubation. This demonstrates that, when the enzyme is only briefly exposed to acid, the optical changes are reversible.

The structure of the prosthetic group of myeloperoxidase remains ambiguous. Resonance Raman spectroscopy data have suggested that the structure of the chromophore is that of a chlorin [14–18]. The asymmetry of the chlorin-like structure has been interpreted to reflect in the resonance Raman spectrum in the oxidation-state marker ($\lambda_x$) region. Due to asymmetry effects multiple lines arise in this region, as can be seen in the spectra of the oxidized and reduced native enzyme (Fig. 3a, trace A and Fig. 3b, trace A). During a short exposure to acid, the resonance Raman spectrum of neither oxidized nor reduced myeloperoxidase were affected. Also, the resonance Raman spectra of the enzyme that was briefly exposed to acid and subsequently reduced and of that which was exposed to acid for a short period after which the pH was increased to pH 7.2 (species R and R in Fig. 5, respectively) are very similar to that of the reduced native enzyme (species R in Fig. 5), whereas the multiple lines in the oxidation-state marker region are of contrast, after a short exposure to acid, the optical absorption spectrum was affected and the Soret band is observed at 448 nm. This proves that a short exposure to acid has no effect on the resonance Raman spectrum, whereas the optical absorption spectrum is strongly affected. This implies that the unusual optical spectrum does not correlate directly with the asymmetric chlorin-like resonance Raman spectrum. Such a situation can arise when the excited electronic-state manifold, but not the ground-state manifold, is selectively perturbed. For example, dimerization of metalloporphyrins can produce strong excitation-coupling effects that shift optical absorption maxima, but not ground-state Raman vibrational frequencies [29].

Our earlier work showed that the EPR spectrum of myeloperoxidase is reversibly affected by exposure to 5 M guanidine·HCl [27]. Similar reversible changes were seen upon exposure of the enzyme to acid and neutralization. The EPR spectrum of the enzyme that was exposed to acid for a short period (species O$_2$ in Fig. 5) displayed a high-spin axial signal (Fig. 2, spectrum B), whereas the spectrum of the enzyme after neutralization (species O$_2$ in Fig. 5) displayed a high-spin signal with rhombic symmetry (Fig. 2, spectrum C). This spectrum had a slightly higher rhombicity relative to that of the native enzyme (cf. Fig. 2, spectrum A and Fig. 2, spectrum C). This is probably due to the presence of chloride, since chloride has been shown to affect the EPR spectrum of native myeloperoxidase [30, 31]. Our results show that the asymmetry in the pyrrole nitrogen atoms can be reversibly removed by exposure to acid. A similar proton-dependent interchange between a high-spin rhombic EPR signal and a high-spin axial EPR signal has been reported for haemoglobin II from *Lucina pectinata* [32]. We suggest, in line with [32], that a protonatable residue in myeloperoxidase is present which imposes the asymmetry on the pyrrole nitrogens. At low pH this residue becomes protonated and the asymmetry in the EPR spectrum is lifted.

From studies on photochemically modified myeloperoxidase, it was concluded that the red-shifted spectrum is not a prerequisite for the chlorinating activity [21]. Our earlier guanidine·HCl denaturation/renaturation experiments, however, suggested that the red-shifted optical spectrum is correlated with the chlorinating activity [27]. From our experiments here on the effect of acid on the spectral properties and the chlorinating activity, we conclude that the chlorinat-
Fig. 4. Long-term effect of acid on the resonance Raman spectra of myeloperoxidase. (a) The spectra were obtained using 20-mW laser power at 413.1 nm and the resonance Raman data were accumulated during 120 s. (A) Myeloperoxidase (40 µM) was incubated in acid for 20 min. (B) Myeloperoxidase (30 µM) was incubated in acid for 20 min after which the pH was increased to pH 7.0. (b) The spectra were obtained using 20-mW laser power at 441.6 nm. Traces (A–C) Myeloperoxidase (40 µM) was incubated in acid for 50 s and the enzyme was reduced. At different time intervals the resonance Raman spectrum was recorded. The resonance Raman data were accumulated during 30 s in A and B, 120 s in C and 180 s in D, respectively. (A) Spectrum after 50 s of incubation. (B) Spectrum after 3 min of incubation. (C) Spectrum after 20 min of incubation. (D) Myeloperoxidase (30 µM) was incubated in acid for 20 min after which the pH was increased to pH 7.0 and the enzyme was reduced.
ing activity is not correlated with the unique spectral properties in line with [21]. Further, prolonged incubation in acid leads to a complete diminishing of the chlorinating activity. We also conclude that peroxidation activity measured by means of the guaiacol assay for 'acid myeloperoxidase' [10], which was reported to be the same for the 'acid myeloperoxidase' and for the native enzyme, is not indicative of the specific activity of myeloperoxidase. That the enzyme slowly inactivates in acid is also clear from the irreversible changes in the optical and resonance Raman spectra.

Prolonged exposure to acid changed the optical spectrum irreversibly and the species formed (O₅ in Fig. 5) had a Soret band at 412 nm and a visible absorption band at 554 nm. The spectrum of this species closely resembles that of oxidized 'acid myeloperoxidase' [10].

Also the recovery of the red-shifted reduced native spectrum (λₒₜₜ in 472 nm) appeared to be increasingly more incomplete when the enzyme was incubated for a longer period in acid and it was not possible to recover the reduced native spectrum. After neutralization, the species (R₄ in Fig. 5) displayed a Soret band at 448 nm. Similarly, upon prolonged exposure to acid, the resonance Raman spectra of both the oxidized (species O₅, O₄ in Fig. 5) and the reduced enzyme (species R₄, R₃ in Fig. 5) were modified. The multiple lines in the oxidation-state marker region in these spectra were replaced with a single symmetrical ν₄ line. The dynamics of this process are illustrated in Fig. 4b, traces A-C. After 20 min of incubation the conversion was complete and this was irreversible. Neutralization of the enzyme solution did not restore the native resonance Raman spectrum (Fig. 4b, trace D). The resonance Raman spectrum (Fig. 4a, trace A) of the oxidized enzyme that was exposed to acid for 20 min (species O₅ in Fig. 5) shows a symmetrical ν₄ line at 1379 cm⁻¹. The corresponding optical spectrum displays the Soret band at 412 nm. Chemically modified ferric myeloperoxidase [21] also displays a Soret band maximum at 412 nm. However, the resonance Raman spectrum of this species is different, with the ν₄ line at 1365 cm⁻¹. In these studies the chromophore was modified and the difference between the Raman spectra (especially the position of the ν₄ lines) may reflect this modification. Furthermore, it should be noted that this chemical modification is irreversible [21].

The optical spectrum of the enzyme that was exposed to acid for 20 min and subsequently neutralized resembles that observed after long exposure to guanidine - HCl [27]. The corresponding resonance Raman spectra were similar with the ν₄ singlet at 1379 cm⁻¹. This indicates that the irreversible effect of acid is reflected in a similar fashion in the resonance Raman spectrum to the irreversible effect of denaturation with guanidine - HCl.

The presence of the stretching frequency of a formyl group in the resonance Raman spectrum, which has been suggested to be present as a peripheral substituent on the macrocycle of myeloperoxidase, could not be detected unambiguously in any of the acid-incubated states. However, it has been shown that the stretching vibration of the formyl group on a macrocycle can be strongly coupled to other vibrational modes, such that the formyl vibrational mode is difficult to detect [33]. This may explain the discrepancy between the chemical detection of a formyl group in myeloperoxidase [5] and the failure to detect clearly its stretching frequency by means of resonance Raman spectroscopy. An exception to this may be the 1642-cm⁻¹ vibration that we observe in species O₄ (Fig. 4a, trace B). The frequency of this mode is characteristic of a formyl group interacting through a hydrogen bond with the local protein environment. Since, in the irreversible first phase, the optical spectrum is strongly affected by the treatment with acid and the resonance Raman spectrum is essentially not affected by this treatment, one may conclude that the irreversible interaction of the protein moiety with the chromophore which is affected by acid and which clearly determines the red shift in the optical spectrum does not affect the resonance Raman spectrum.

### Table: Spectral Changes

| Species | Optical Spectrum | Activity | EPR | RR
|---------|------------------|---------|-----|---
| Reduced R₂ | 472 nm, 636 nm | + | ε⁻ (reduction) | 1329 / 1353 / 1379 cm⁻¹
| Reduced R₁ | 448 nm, 505 nm | + | ε⁻ (reduction) | 1325 / 1353 / 1379 cm⁻¹
| Oxidized O₁ | 430 nm, 570 nm | + | ε⁻ (reduction) | 1367 / 1379 cm⁻¹
| Oxidized O₂ | 428 nm, 570 nm | + | ε⁻ (reduction) | 1367 / 1379 cm⁻¹
| Oxidized O₃ | 412 nm, 554 nm | + | ε⁻ (reduction) | 1379 / 1394 cm⁻¹
| Oxidized O₄ | 428 nm, 590 nm | + | ε⁻ (reduction) | 1379 / 1394 cm⁻¹

Fig. 5. Overview of the properties of various myeloperoxidase intermediates observed during the incubation in acid and their interconversion. RR = resonance Raman.
Our experiments show that it is possible to modulate the optical spectrum of myeloperoxidase between a blue-shifted spectrum and the peculiar red-shifted spectrum by changing the pH. This indicates that a specific interaction of the protein moiety with the prosthetic group is responsible for the red-shifted optical absorption spectrum, as has been suggested before [4]. Charges close to a porphyrin in a protein-porphyrin complex may significantly affect the positions of the absorption maxima of the protein. Theoretical considerations have shown that charges in the vicinity of chlorophyll may significantly affect the position of the absorption maxima [34]. It has been suggested, in view of these calculations, that a protonatable, negatively charged residue near the chromophore (0.3–0.35 nm) may be present in myeloperoxidase, which may become protonated at low pH and which affects the electronic structure of the prosthetic group and imposes the red shift in the absorption spectra [27]. The present experiments with acidic treatment fully support this suggestion. At low pH, the negatively charged residue may become protonated and thus unchanged. It is not possible to titrate this protonatable group because the spectral changes are time-dependent and a slower irreversible process also occurs at low pH. The strong red shift in the optical spectrum of reduced myeloperoxidase is reversibly lifted by treatment with acid, which does not affect the ground-state Raman vibrational frequencies. This can also be rationalized by an interaction of the protein environment with the chromophore, which is susceptible to acid, such as protonation of carboxylic residues. Such a protonation reaction could affect the excited electronic state manifold, without perturbing the ground-state geometry and the resonance Raman spectrum significantly.

It has indeed been shown [8] that the carboxylic groups of Asp94 and Glu242 are very close (0.3–0.4 nm) to the chromophore. These residues may impose the red shift in the optical spectrum and cause the asymmetry on the pyrrole nitrogen atoms from the macrocyclic ring coordinating the iron. These residues are buried within the protein matrix and cannot interact directly with substrates, such as chloride and hydrogen peroxide, and this may explain the fact that the red-shifted optical spectrum is not correlated with the chlorinating activity of the enzyme.

Site-directed mutagenesis studies [35], which are on the way, will show whether this suggestion is correct.

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REFERENCES


