On the unusual heme group of myeloperoxidase

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Difference Fourier transform infrared evidence for ester bonds linking the haem group in myeloperoxidase, lactoperoxidase and eosinophil peroxidase

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Abstract

The homologous mammalian peroxidases contain a haem group, which is believed to form covalent linkages to the protein moiety via two ester bonds. Myeloperoxidase is distinguished from the other mammalian peroxidases by the presence of an additional sulphonium ion linkage, involving Met243, which is probably responsible for its unique spectroscopic and enzymatic characteristics. Difference Fourier transform infrared spectroscopy provides evidence for the presence of the ester bonds linking the haem group to the protein in myeloperoxidase, lactoperoxidase and eosinophil peroxidase. Two sets of carboxylic ester C=O and C-O- group absorptions were observed, and site-directed mutagenesis of myeloperoxidase allowed them to be assigned specifically to Glu242 and Asp94. Mutations of Met243, addition of halides or prolonged exposure to acid mainly affect the positions of the Asp94 ester bond.
FTIR detection of haem-linking ester bonds

Introduction

The homologous mammalian peroxidase family comprises myeloperoxidase (MPO), lactoperoxidase (LPO), eosinophil peroxidase (EPO) and thyroid peroxidase (TPO). MPO shares respectively 61, 70 and 47% identical residues with these peroxidases (Kimura & Ikeda-Saito, 1988, Sakamaki et al., 1989, Ueda et al., 1997), and an even higher homology can be found among the active site related residues. MPO differs significantly from other peroxidases in its unusual spectral features (Newton et al., 1965b, Wever & Plat, 1981) and its unique ability to catalyse the oxidation of chloride by hydrogen peroxide to form the potent oxidant and bactericidal agent hypochlorous acid (Harrison & Schultz, 1976). These differences have been attributed at least partly to the chemical properties of its haem group. Since the haem group is covalently linked to the protein, its characterisation has been difficult. Based on the results of spectroscopic studies (optical absorbance, resonance Raman, magnetic circular dichroism and others) and chemical studies, a formyl containing haem α-like structure (Newton et al., 1965b, Odajima, 1980, Sono et al., 1991), a chlorin-like haem structure (Babcock et al., 1985, Eglinton et al., 1982, Sibbett & Hurst, 1984), and an iron protoporphyrin-like structure (Floris et al., 1995) have been proposed. Fenna et al. (Fenna et al., 1995) reported a crystal structure at 2.28 Å resolution for human MPO and suggested that the haem is a novel derivative of iron protoporphyrin IX which forms three covalent bonds with the protein. Hydroxylated methyl groups on pyrrole rings A and C were claimed to form ester linkages with Glu242 and Asp94, while a covalent bond between the vinyl group on ring A and the sulphur atom of Met243 was proposed to be a sulphonium ion linkage (Fenna et al., 1995, Taylor et al., 1995), which is probably the origin of its unique characteristics (Kooter et al., 1997a). On the basis of NMR and mass spectrometric studies of the haem (Rae & Goff, 1996) and spectral analysis (Andersson et al., 1996) a similar structure has been proposed for LPO in which the sulphonium ion linkage is absent. The corresponding ester forming glutamate and aspartate residues are conserved throughout the mammalian peroxidase family (Zeng & Fenna, 1992). Recently DePillis et al. (DePillis et al., 1997) provided conclusive evidence that the prosthetic group of LPO was a haem, which in the presence of H₂O₂ is autocatalytically esterified to the protein. It has been suggested that a

Table 1. Frequencies* of carbonyl containing groups.

<table>
<thead>
<tr>
<th>Carboxylate Ions</th>
<th>1610-1555 cm⁻¹ (asymmetric)</th>
<th>1420-1300 cm⁻¹ (symmetric)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esters</td>
<td>1800-1650 cm⁻¹</td>
<td>1310-1100 cm⁻¹</td>
</tr>
<tr>
<td>Carboxylic Acids</td>
<td>1740-1650 cm⁻¹</td>
<td>1400, 1250, 920 cm⁻¹</td>
</tr>
</tbody>
</table>

* frequency data from Bellamy (Bellamy, 1956).
common feature (Andersson et al., 1996) of all mammalian peroxidases is this covalent linkage via two ester bonds.

Carbonyl stretching vibrations are readily detected by Fourier transform infrared (FTIR) spectroscopy (Table 1). In proteins carbonyl stretches due to the carboxylic acid side chains of aspartate (Dioumaev & Braiman, 1995, Engelhard et al., 1985) or glutamate residues (Xie et al., 1996), as well as of esters on bacteriochlorophyll a (Hanwich et al., 1995), have been reported. Here we report the results of FTIR difference spectroscopy studies of MPO, LPO and EPO, and MPO mutants. These studies provide the first direct spectroscopic evidence for the presence of two distinguishable ester groups, for which Asp94 and Glu242 are responsible.

Materials and methods

Transfection of recombinant plasmids into Chinese hamster ovary cells, selection and culture procedures for transfected cells, protein purification protocols, Western blotting, ELISA, and electrophoretic analysis of recombinant MPO were described in detail previously (Moguilevsky et al., 1991). The Asp94Val, Glu242Gln and Met243Thr mutants were produced by replacing, in the MPO-coding cassette carried by plasmid pNIV2703, a 178 bp Apal-Avrll DNA fragment by the mutated counterpart. The final plasmids were called pNIV2729, 2714 and 2719 respectively. The mutation was generated within this fragment by a combination of polymerase chain reactions and overlap extensions, using an oligonucleotide primer carrying the modified codon. The amplified fragment was sequenced using Sequenase version 2 (U.S. Biochemical Corp.). The final recombinant plasmid was transfected into Chinese hamster ovary cells, and cell factories supernatant (10 l) was collected, and the mutant was purified (Moguilevsky et al., 1991).

MPO and EPO samples were purified from human leukocytes using the published procedures (Bakkenist et al., 1978, Wever et al., 1981). LPO sample was purified from whey (Paul et al., 1980).

IR spectra were recorded on a Bio-Rad FTS-60A FTIR spectrometer equipped with a KBr beamsplitter and a MCT detector. All measurements were carried out with a home-made 'sandwich' IR-cell composed of two CaF2 plates separated by a 14 μm polyethylene spacer (prepared from a sandwich bag). Spectra were obtained by first recording a single beam spectrum of the oxidised form of the sample containing 2.5 mM deazaflavin and 25 mM EDTA. Then the sample was photo-reduced (Lübken & Gerwert, 1996, Tollin, 1995) by exposure to visible light from a 150 W Oriel Xenon lamp, via an optical fibre, and a reduced-oxidised spectrum was recorded. The spectra were corrected for water vapour and recorded at room temperature. The oxidation state of the sample in the FTIR cell was monitored by visible spectroscopy using a Hewlett-Packard 8452 A diode array spectrophotometer. Although additional difference bands were detected both in the 3000-2500 and 1700-1000 cm⁻¹ regions, we focus here on the 1800-1700 and 1200-1100 cm⁻¹ regions.
Results and discussion

Reduced-oxidised FTIR difference spectra (Mäntele, 1993) of human MPO, bovine LPO and human EPO are shown in Figure 1. The most prominent features for MPO are negative bands (derived from the oxidised enzyme state) at 1756 and 1728 cm\(^{-1}\) and a positive band (derived from the reduced enzyme state) at 1742 cm\(^{-1}\). Stepwise reduction of the sample and careful analysis of the spectra revealed an additional weak positive band at 1723 cm\(^{-1}\), which is occasionally obscured by baseline drift. In the lower frequency region two negative bands at 1164 and 1152.5 cm\(^{-1}\) and two positive bands at 1170 and 1159 cm\(^{-1}\) could be observed, with intensities comparable to those in the 1800-1700 cm\(^{-1}\) region. Bovine LPO also shows two negative bands at 1754 and 1735 cm\(^{-1}\), and two positive bands at 1742 and 1730 cm\(^{-1}\). At lower frequency one main negative band at 1155 and one main positive band at 1171 cm\(^{-1}\).

![Figure 1. Reduced-oxidised FTIR difference spectra of MPO, LPO (×2) and EPO (×5). The samples (1.4, 1.5 and 0.3 mM, respectively) were in 50 mM potassium phosphate buffer with 25 mM EDTA and 2.5 mM deazaflavin, pH 7.0. Each spectrum is the sum of 381, 762 and 762 scans respectively, with 2 cm\(^{-1}\) resolution.](image)

was observed, again with similar intensity compared to the bands in the 1800-1700 cm\(^{-1}\) region and to MPO. In EPO, which was hard to obtain in concentrated form due to its tendency to aggregate, two negative bands at 1755 and 1737 cm\(^{-1}\) and two positive bands at 1742 and 1734 cm\(^{-1}\) could be detected. In the lower frequency region it was difficult to assign negative or positive bands.
The position of the observed bands precludes their assignment to -COO$^-$ groups (Table 1), since the carbonyl stretches of carboxylate ions are found at 1610-1550 cm$^{-1}$ (asymmetric stretch) and 1420-1300 cm$^{-1}$ (symmetric stretch) (Bellamy, 1956). The remaining possibilities for the bands in the 1800-1700 cm$^{-1}$ region are that they arise from ester or COOH group vibrations. Esters have two characteristic strong absorptions arising from the C=O and C-O- groups (Table 1), usually found in the 1800-1650 cm$^{-1}$ and 1310-1100 cm$^{-1}$ region, respectively (Bellamy, 1956). COOH groups show a strong absorption band in the 1740-1650 cm$^{-1}$ region due to the C=O stretch, and bands near 1400, 1250, and 920 cm$^{-1}$, which are arising from the C-O- stretching vibrations and to the OH deformation mode (Bellamy, 1956). Frequencies above 1740 cm$^{-1}$ are rare for carboxylic acids and have only been reported for non-hydrogen bonded carboxylic acids (Dioumaev & Braiman, 1995). On the basis of the position and intensities of the observed bands in both 1800-1700 and 1200-1100 cm$^{-1}$ region, we can assign them to ester groups. This is confirmed by control experiments in which FTIR difference spectra were recorded for MPO that was extensively equilibrated in D$_2$O, including a reduction-oxidation cycle (Figure 2). In none of these experiments were downshifted bands observed, as would have been expected for COOD groups. Instead a small upshift of 1 cm$^{-1}$ is detected, possibly due to a minor change in secondary structure upon deuteration (Uno et al., 1987).

![Figure 2](image)

**Figure 2.** Reduced-oxidised FTIR difference spectra of wild-type MPO in H$_2$O and D$_2$O solutions. The samples (1.4 and 1.3 mM, and 381 and 762 scans, respectively) were measured as in Figure 1.
With the help of site-directed mutagenesis, we were able to unambiguously assign the bands in the 1800-1700 cm$^{-1}$ region to the ester carbonyls and the bands in the 1200-1100 cm$^{-1}$ region to the -C-O- stretch of two specific residues (Figure 3). Mutation of Glu242 of MPO into glutamine results in the loss of the 1728, 1723, 1164 and 1170 cm$^{-1}$ bands (Figure 3), which demonstrates conclusively that these spectral features arise from an ester group derived from Glu242. The negative band at 1757 cm$^{-1}$ and the positive band at 1742 cm$^{-1}$, as well the negative band at 1152.5 cm$^{-1}$ and the positive band at 1159 cm$^{-1}$ are essentially unchanged by the mutation. Figure 3 also shows the spectrum of a mutant in which Asp94 of MPO is replaced by valine. In line with the observations on the Glu242Gln mutant, this mutation results in the loss of both the 1756 and 1742 cm$^{-1}$ bands and the 1152.5 and 1159 cm$^{-1}$ bands in the reduced-oxidised FTIR difference spectrum. The original 1728 cm$^{-1}$ band exhibits a minor upshift of 2 cm$^{-1}$, whereas the 1164 and 1170 cm$^{-1}$ bands are unchanged.

This result demonstrates conclusively that the second set of features observed arise from an ester group derived from Asp94. Our results are not consistent with the earlier proposal that Glu242 and Asp94 influence the haem group via electrostatic effects due to the negatively charged carboxylate residues (Floris et al., 1994, Floris et al., 1995). Instead, our data demonstrate that the two carboxylic acid residues are covalently attached to the hydroxylated haem group by esterification.

![Graph showing FTIR difference spectra](image-url)
A revised structure for the chromophore of MPO that is consistent with these data is shown in Figure 4. The presence of a bond between the methionine sulphur atom and the $\alpha$-carbon of the vinyl group rather than a unprecedented vinyl sulphonium ion (Taylor et al., 1995), is proposed by analogy to the chemistry involved in formation of the thioether groups that are present in cytochromes c.

![Figure 4. Proposal for the haem structure of MPO, based on (Taylor et al., 1995), but modified with respect to the details of the sulphonium ion linkage.](image)
In order to investigate the influence of the sulphonium ion linkage on the ester bonds, we mutated Met243 into threonine. This Met243Thr mutant is not able to make the proposed sulphonium ion linkage to the haem group, and spectroscopic and catalytic properties have drastically changed compared to the wild-type MPO (Chapter 5). Mutation of Met243 into Thr results in the loss of the two distinguishable ester bands in the 1800-1700 cm\(^{-1}\) region (Figure 5), instead one main negative feature at 1746 cm\(^{-1}\) is observed, no clear positive band is observed. In the lower frequency region one main negative band at 1161 cm\(^{-1}\) and one main positive band at 1171 cm\(^{-1}\) is observed, corresponding well with the C-O- stretch of the Glu242 ester bond in wild-type MPO. It is not clear whether only one ester bond is present, or that the frequencies of the distinguishable ester bonds have shifted to similar positions, leading to partial cancellation of the bands. However, the position of the ester bands is influenced considerably in the Met243Thr mutant.

\[ \text{Figure 5. Reduced-oxidised FTIR difference spectra of wild-type MPO and Met243Thr mutant (x2). The samples (1.4 and -0.7 mM, 381 and 762 scans, respectively) were measured as in Figure 1.} \]
Halides are known to interact with MPO to give spectroscopically distinguishable complexes (Bakkenist et al., 1980, Bolscher & Wever, 1984). Figure 6 shows the effect of fluoride and chloride on the position of the ester carbonyl and -C-O- stretches. Upon addition of halide the negative band at 1756 cm\(^{-1}\) is shifted 10 cm\(^{-1}\) to lower frequency. In the lower frequency region one main negative band at 1166 cm\(^{-1}\) is observed. This band corresponds well with that found for the C-O- stretch of the Glu242 ester bond of MPO in the absence of halide. The intensity of this band suggests that the C-O- stretch of the Asp94 ester has shifted to a position similar of the Glu242 ester bond. The positions of the Asp94 ester bond derived frequencies are most affected by addition of halide.

![Figure 6. Reduced-oxidised FTIR difference spectra of wild-type MPO (x0.5), its chloride bound form and fluoride bound form. The samples (1.4, 0.6 and 0.8 mM, respectively and 762 scans), contained 0.1 M KF or NaCl and were in 0.1 M sodium citrate buffer (pH 5.0) and were measured as in Figure 1.](image)
Prolonged (20 min.) exposure to acid has been shown to irreversibly affect the optical absorbance and resonance Raman spectra, and the chlorinating activity of MPO (Floris et al., 1994). It was proposed that a negatively charged protonatable residue, like Asp94 or Glu242, in the proximity of a pyrrole nucleus of the haem group is present, that imposes the red-shift in the optical absorption spectrum (Floris et al., 1994). Since we know now that Asp94 and Glu242 are involved in ester bonds to the haem group, we examined this incubation of enzyme in acid by FTIR. Figure 7 shows MPO prior to 19 mM HCl incubation, in 19 mM HCl and after neutralisation. Incubation (30 min.) in 19 mM HCl shifts the position of the carboxyl stretch of the Asp94 ester bond in its oxidised state, by 8 cm$^{-1}$ to lower frequency. The bands at 1742 cm$^{-1}$, 1728 cm$^{-1}$, and 1723 cm$^{-1}$ are unaffected. In the lower frequency region one main positive band at 1170 cm$^{-1}$ and one main negative band at 1164 cm$^{-1}$ are observed. Neutralisation of the sample after 30 minutes incubation in 19 mM HCl results in the original spectrum. This clearly demonstrates that incubation in 19 mM HCl does not irreversibly affect the ester bonds.

![Figure 7. Long term effect of acid on the reduced-oxidised FTIR difference spectra of MPO. (A) wild-type MPO, (B) MPO incubated in the presence of 19 mM HCl for 30 min. (x2), (C) MPO incubated in the presence of 19 mM HCl for 30 min. after which the pH was increased to pH 7.0 with 0.1 M potassium phosphate (x2). The samples (1.1, 0.7 and 0.6 mM, respectively and 762 scans) were measured as in Figure 1.](image-url)
Conclusions

I. The mammalian peroxidases, MPO, EPO, and LPO contain ester bonds between hydroxylated methyl groups of the haem and the protein. With help of site-directed mutagenesis we have shown that the Asp94 and Glu242 residues in MPO are responsible for these two distinguishable ester bonds.

II. Mutation of Met243, involved in the sulphonium ion linkage, affects the vibration frequencies of the ester bonds.

III. Addition of halides, like fluoride and chloride, affects mainly the frequency positions of the Asp94 ester bond.

IV. Incubation of the enzyme in 19 mM HCl (30 min.) reversibly affects the frequency positions of the Asp94 ester bond.

V. We have shown that difference FTIR spectroscopy can be a useful technique to unravel structural properties of haem proteins.

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Conclusions

The maximum predicted MPO, EPO, and LPO-controls after bonds between 1-dodecyl-2-thiol groups on the bond and the protein. With help of the directed acyclic graph, we found that the 
MQR54 and XMR547 residues on MPO are responsible for these without covalent bonds.

Effects at MQR54, involved in the stabilization of the forms, affects the stabilization frequency of the other bonds.

Inhibition of enzyme inhibition of the two forms affects the frequency of the residues.

Further work is required to understand the role of these residues in the frequency of the MPO and LPO-controls.