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Difference Fourier Transform Infrared Evidence for Ester Bonds Linking the Heme Group in Myeloperoxidase, Lactoperoxidase, and Eosinophil Peroxidase

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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,[1−3] 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[4,5] and its unique ability to catalyze the oxidation of chloride by hydrogen peroxide to form the potent oxidant and bactericidal agent hypochlorous acid.[6] These differences have been attributed at least partly to the chemical properties of its heme group. Since the heme group is covalently linked to the protein, its characterization has been difficult. On the basis of the results of spectroscopic studies (optical absorbance, resonance Raman, magnetic circular dichroism, and others) and chemical studies, a formyl containing heme *a*-like structure,[5,7,8] a chlorin-like heme structure,[9−11] and an iron protoporphyrin-like structure[12] have been proposed. Fenna et al.[13] reported a crystal structure at 2.28 Å resolution for human MPO and suggested that the heme is a novel derivative of 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 sulfur atom of Met243 was proposed to be a sulfonium ion linkage,[13,14] which is probably the origin of its unique characteristics.[15] On the basis of NMR and mass spectrometric studies of the heme[16] and spectral analysis,[17] a similar structure has been proposed for LPO in which the sulfonium linkage is absent (corresponding ester forming glutamate and aspartate residues are conserved throughout the mammalian peroxidase family[18]). Recently DePilles et al.[19] provided conclusive evidence that the prosthetic group of LPO was a heme which in the presence of H_2O_2 is autocatalytically esterified to the protein. It has been suggested that a common feature[17] of all mammalian peroxidases is this covalent linkage via two ester bonds.

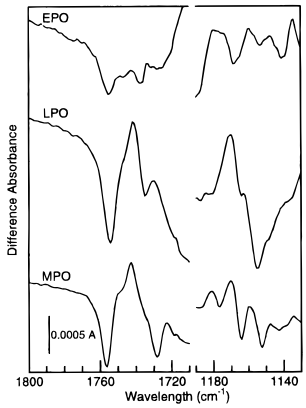

Figure 1. Reduced−oxidized FTIR difference spectra of MPO, LPO (×2), and EPO (×5). The samples (1.4, 1.5, and 0.3 mM, respectively) were in 50 mM potassium phosphate buffer with 25 mM EDTA and 2.5 mM deazaflavin (pH 7.0). Each spectrum is the sum of 381, 762, and 762 scans, respectively, with 2 cm^{-1}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

Carbonyl stretching vibrations are readily detected by Fourier transform infrared (FTIR) spectroscopy. In proteins carbonyl stretches due to the carboxylic acid side chains of aspartate[20,21] or glutamate residues,[22] as well as of esters on bacteriochlorophyll *a*,[23] 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.

Reduced-oxidized FTIR difference spectra[24,25] of human MPO, bovine LPO, and human EPO[26] are shown in Figure 1. The most prominent features for MPO are negative bands (derived from the oxidized enzyme state) at 1756 and 1728 cm^{-1} and a positive band (derived from the reduced enzyme state) at

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(25) IR spectra were recorded on a Bio-Rad FTS-60A FTIR spectrometer equipped with a KBr beam splitter and a MCT detector. All measurements were carried out with a homemade "sandwich" IR-cell composed of two CaF_2 plates separated by a 13.5 μm polyethylene spacer (prepared from a sandwich bag). Spectra were obtained by first recording a single beam spectrum of the oxidized form of the sample containing 2.5 mM deazaflavin and 25 mM EDTA. Then the sample was photoreduced[31,32] by exposure to visible light from a 150 W Oriel Xenon lamp, via an optical fiber, and a reduced-oxidized spectrum was recorded. The spectra were corrected for water vapor 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.
(26) MPO and EPO samples were purified from human leukocytes using the published procedures.[33,34] The LPO sample was purified from whey.[35]

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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} was observed, again with intensities similar to those of the bands in the 1800−1700 cm^{-1} region and to that of 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, since the carbonyl stretches of carboxylate ions are found at 1610−1550 cm^{-1} (asymmetric stretch) and 1420−1300 cm^{-1} (symmetric stretch).[27] 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, usually found in the 1800−1650 cm^{-1} and 1310−1100 cm^{-1} regions, respectively.[27] 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 assigned to the C−O− stretching vibrations and to the OH deformation mode.[27] Frequencies above 1740 cm^{-1} are rare for carboxylic acids and have only been reported for non-bonded carboxylic acids.[21] On the basis of the positions and intensities of the observed bands in both 1800−1700 and 1200−1100 cm^{-1} regions, we can assign them to ester groups. This is confirmed by control experiments in which FTIR difference spectra were recorded for MPO which was extensively equilibrated in D_2O, including a reduction−oxidation cycle (not shown). 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, presumably due to a minor change in secondary structure upon deuteration.[28]

With the help of site-directed mutagenesis,[29] 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 2). Mutation of glutamate 242 of MPO into glutamine results in the loss of the 1728, 1723, 1164, and 1170 cm^{-1} bands (Figure 2), which demonstrates conclusively that these spectral features arise form 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 2 also shows the spectrum of a mutant in which aspartate 94 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-oxidized 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 glutamate 242 and aspartate 94 influence the heme group via electrostatic effects due to the negatively charged carboxylate residues.[12,30] Instead, our data demonstrate that the two carboxylic acid residues are covalently attached to the hydroxylated heme group by esterification.

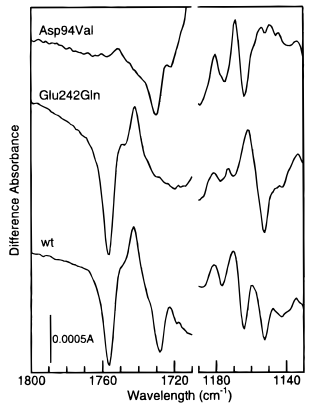

Figure 2. Reduced−oxidized FTIR difference spectra of wild-type MPO, Glu242Gln mutant MPO (×2), and Asp94Val mutant MPO (×3). The samples (1.4, 0.5, and 0.7 mM) were measured as in Figure 1.

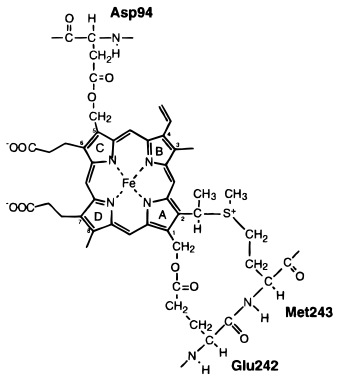

Figure 3. Proposal for the heme structure of MPO, based on,[14] but modified with respect to the details of the sulfonium ion linkage.

In this study, we have shown for the first time that FTIR difference spectroscopy can be used to detect the ester linkages between the hydroxylated heme group and the protein carboxyl groups in the mammalian peroxidases MPO, LPO, and EPO (and presumably also TPO). A revised structure for the chromophore of MPO that is consistent with these data is shown in Figure 3. The presence of a bond between the methionine sulfur atom and the α-carbon of the vinyl group rather than a unprecedented vinyl sulfonium ion,[14] is proposed by analogy to the chemistry involved in formation of the thioether groups that are present in cytochrome *c*.

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