On the unusual heme group of myeloperoxidase

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The sulphonium ion linkage in myeloperoxidase: direct spectroscopic detection by isotopic labelling and effect of mutation

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The haem group of myeloperoxidase is covalently linked via three amino acid side chains to the protein. The Asp94 and Glu242 form ester bonds to the haem, whereas the third residue Met243, forms a unique sulphonium ion linkage to the haem group. In this study we show that this sulphonium ion linkage is responsible for the unique characteristics of MPO. Mutation of Met243 into Thr, Gin and Val, which are the corresponding residues of eosinophil peroxidase, lactoperoxidase and thyroid peroxidase respectively, and into Cys were performed. The Soret band in the optical absorbance spectrum in the oxidised mutants is now found at approximately 411 nm, a position similar to that found for the other mammalian peroxidases. The pyridine haemochrome spectra show that the chemical nature of the haem group is affected by the mutation. Resonance Raman spectra of the mutants are less complicated than those of the wild-type MPO, and are indicative of an iron protoporphyrin prosthetic group. In the Met243 mutants the chloride binding properties are greatly affected, and the dissociation constants (K_d) have increased 100-fold. All mutants have lost their chlorination activity, except for the Met243Thr mutant, which still has 15% activity left. By FTIR difference spectroscopy it was possible to specifically detect the 13CD3-labelled methionyl sulphonium linkage.

We conclude that the sulphonium ion linkage serves two roles. First, it serves as an electron-withdrawing substituent via its positive charge, and, second, together with its neighbouring residue Glu242, it appears to be responsible for the lower symmetry of the haem group and distortion from the planar conformation normally seen in haem-containing proteins.
Introduction

In the family of mammalian peroxidases, myeloperoxidase (MPO) is an extraordinary peroxidase. First of all, the enzyme is the only mammalian peroxidase known to peroxidise chloride to hypochlorous acid at a substantial rate. Secondly, MPO differs in its spectroscopic characteristics by its unusual red-shifted Soret band in the optical absorbance as well in its pyridine haemochrome spectrum, its complicated resonance Raman spectrum, and its inverted sign-pattern of the Soret band in the MCD spectrum (Agner, 1941, Babcock et al., 1985, Eglinton et al., 1982, Newton et al., 1965b, Sibbett & Hurst, 1984, Sono et al., 1986). Those differences have been attributed to the special nature or structure of the haem group in MPO. Since this haem is covalently bound to the protein, characterisation of this chromophore has been difficult. Based on different spectroscopic techniques, a formyl-containing haem α, a chlorin, or a haem b prosthetic group have been proposed (Dugad et al., 1990, Eglinton et al., 1982, Harrison & Schultz, 1978, Schultz & Shmukler, 1964, Sibbett & Hurst, 1984).

Although the enzyme differs in spectroscopic and catalytic properties the homology between MPO and the other mammalian peroxidases is high. MPO shares respectively 70, 61, and 47% identical residues with eosinophil peroxidase (EPO), lactoperoxidase (LPO) and thyroid peroxidase (TPO) (Kimura & Ikeda-Saito, 1988, Sakamaki et al., 1989, Ueda et al., 1997), and an even higher homology can be found among the residues in the active site. MPO is the only mammalian peroxidase for which a crystal structure is known, at 2.3 Å resolution (Fenna et al., 1995). The structural data for human MPO suggested that three haem substituents form covalent bonds with amino acid side chains in the protein. Two ester bonds were claimed to be present, between modified methyl groups on pyrrole rings A and C and the amino acids Glu242 and Asp94. In a recent study, we have provided the first direct evidence by FTIR difference spectroscopy that ester bonds link the haem groups in all mammalian peroxidases via the conserved aspartate and glutamate residues (Kooter et al., 1997b). The third linkage involves the non-conserved Met243 residue, for which there is considerable evidence. Based on the unique autocleavage Met243-Pro244 site of MPO, resembling the cyanogen bromide dependent-cleavage of Met-X bonds and the fact that Met243 is in close proximity to the prosthetic group of MPO, Taylor et al. (Taylor et al., 1992a) proposed that Met243 was involved in a sulphonium ion linkage to the haem group. Later studies showed that this sulphonium ion linkage involved the vinyl group of pyrrole ring A (Fenna et al., 1995, Taylor et al., 1995).

Met243 is replaced by a threonine in human EPO (Sakamaki et al., 1989), whereas in bovine LPO a glutamine is found at this position (Cals et al., 1991), and, as recently shown by Ueda et al. (Ueda et al., 1997), in human LPO a histidine is present. It should be mentioned that the genetic codes of Gln and His only differ by one base for both residues. Human TPO has been shown to contain a valine at this position (Kimura et al., 1987). In a recent study (Kooter et al., 1997a), we have mutated the Met243 of MPO into a glutamine, in order to create an LPO-like protein. This mutant MPO is spectroscopically very similar to LPO, and we concluded that Met243 is responsible for the spectral characteristics of MPO.

In this study we further investigated the role of the Met243 residue by mutating it to the corresponding residues of the other mammalian peroxidases, i.e. a threonine for EPO and a valine for TPO. We also mutated the Met243 residue into a cysteine in order to investigate if this residue would still be able to make a linkage to the haem group via its sulphur atom, in analogy with haem c. These mutations of the Met243 residue in MPO resulted in loss of the typical MPO enzymatic and spectroscopic characteristics. The binding of chloride to the
Met243 mutants is also affected; the affinity for Cl\(^-\) has decreased approximately 100-fold. We also show for the first time that it is possible, by vibrational spectroscopy, to detect a single methionine residue in a protein by isotopically labelling of the recombinant MPO.

**Materials and methods**

Transfection of recombinant plasmids into Chinese hamster ovary (CHO) cells, selection and culture procedures for transfected cells, protein purification protocols, Western blotting, ELISA, and electrophoretic analysis of recombinant myeloperoxidase were described in detail previously (Moguilevsky et al., 1991). The Met243Cys, Met243Thr, Met243Gln and Met243Val mutant proteins were produced by replacing, in the myeloperoxidase-coding cassette carried by plasmid pNIV2703, a 178 bp Apal-AvrII DNA fragment by the mutated counterpart. The final plasmids were called pNIV2721, 2719, 2718 and 2717 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 CHO cells, and G418 resistant colonies were selected and expanded. Cell factories supernatant (10 l) was collected, and the mutant was purified (Moguilevsky et al., 1991). The \(^{13}\)CD\(_3\)-labelled methionine recombinant and Met243Thr mutant were produced similarly to the ordinary recombinant and Met243Thr mutant, but the CHO cells were grown in the presence of 1 gram of \(^{13}\)CD\(_3\)-labelled methionine (Isotec, Inc.).

It was found that the recombinant MPO had a lower \(R_z\) value (A\(_{428\text{nm}}\)/A\(_{280\text{nm}}\) \(^{-1}\)) than native MPO, and the mutants also showed different \(R_z\) values. This made it difficult to assess the protein concentration. We therefore determined the concentration of recombinant MPO from the optical absorbance of the Soret band at 428 nm and that of the mutants at their Soret maximum, using in both cases the extinction coefficient of 89 mM \(^{-1}\) cm \(^{-1}\) of native MPO.

All optical spectra for the recombinant and mutants were recorded on a Cary 50 Biospec spectrophotometer. An appropriate amount of dithionite solution was used for reduction. The pyridine haemochrome spectra were prepared in 2.1 M pyridine and 75 mM NaOH, and a concentrated dithionite solution was added for reduction. EPR measurements at X-band were obtained with a Bruker ECS 106 EPR spectrometer at a field modulation frequency of 100 kHz. Cooling of the sample was performed with an Oxford Instruments ESR 900 cryostat with an ITC4 temperature controller. The magnetic field was calibrated with an AEG magnetic field meter. The microwave frequency was measured with an HP 5350 B frequency meter. The resonance Raman spectra were recorded using a confocal Raman microspectrophotometer which was adapted for the experiment using 413.1 nm excitation, as reported before (Kooter et al., 1997a). IR spectra were recorded on a Bio-Rad FTS-60A FTIR spectrometer equipped with a KB\(_r\) beamsplitter and a MCT detector. All measurements were carried out with a home-made 'sandwich' IR-cell composed of two CaF\(_2\) plates separated by a 56 \(\mu\)m polyethylene spacer. \(^{13}\)CD\(_3\)-methionine and methylated-\(^{13}\)CD\(_3\)-methionine solutions were in H\(_2\)O. Methylated-\(^{13}\)CD\(_3\)-methionine was prepared according to Toennies and Kolb (Toennies & Kolb, 1945). Solid state samples were air-dried on a single CaF\(_2\) plate. Difference 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 (Lübben & Gerwert, 1996, Tollin, 1995) by exposure to visible light from a 150 W Oriel Xenon lamp, via an optical fiber and a reduced minus 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.

The chlorinating activity was measured by monitoring the conversion of monochlorodimedone (1,1-dimethyl-4-chloro-3,5-cyclohexanedione) at 290 nm (ε = 20.2 mM⁻¹ cm⁻¹ (Hewson & Hager, 1979)) into dichlorodimedone (ε = 0.2 mM⁻¹ cm⁻¹ at 290 nm). The chlorinating activity was also measured by monitoring the formation of taurine monochloramine at 252 nm (ε = 429 M⁻¹ cm⁻¹ (Thomas et al., 1986)). The guaiacol assay was performed by monitoring the formation of tetraguaiacol at 470 nm (ε = 26.6 mM⁻¹ cm⁻¹ (Chance & Maehly, 1955)). The 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) assay was performed by monitoring the formation of the oxidation product at 414 nm (ε = 36.0 mM⁻¹ cm⁻¹ (Childs & Bardsley, 1975)). The assays were performed with 2.5 mM ABTS, 8 mM guaiacol, 50 µM MCD or 15 mM taurine, respectively. All assays were performed in 100 mM potassium phosphate (pH 7) or sodium acetate (pH 5) and 200 mM Na₂SO₄, and with 5-50 mM MPO and 100 µM H₂O₂. The chlorinating activity assays were performed in the presence of 100 mM NaCl, and the reactions were started by addition of enzyme. The ABTS and guaiacol activity assays were started by addition of H₂O₂. We decided not to explore the kinetic parameters (Vₘₐₓ and Kₘ) values in detail considering the complex kinetic behaviour of MPO (Bakkenist et al., 1980).

For the chloride binding studies the optical absorbance spectrum was recorded before and after the addition of chloride. After correction for volume changes the dissociation constants were calculated from saturation curves of the chloride-induced spectral changes (Bakkenist et al., 1980). Binding studies were performed on an Aminco DW2000 spectrophotometer and a Cary 50 Biospec spectrophotometer.

Results

Here we present a spectroscopic and enzymatic characterisation of all Met243 mutants available; for comparison, LPO data are also shown. Figure 1 shows the optical absorbance spectra of both the oxidised and reduced forms of the Met243Cys, Met243Thr, Met243Gln and Met243Val mutants, LPO, and recombinant MPO. The Soret band in the oxidised enzyme state was found at 410 nm for Met243Cys, 414 nm for Met243Thr, 410 nm for Met243Gln, and at 414 nm for Met243Val mutant, and at 412 nm for LPO, and at 428 nm for recombinant MPO. It was not possible to completely reduce the Met243 mutants at pH 7 by addition of dithionite under anaerobic conditions. Reduction was facilitated by addition of 0.5 µM methylviologen. The Soret band was found at 432 nm for Met243Cys, 447 nm for Met243Thr, first at 445 nm and finally at 430 nm for Met243Gln, and at 414 nm for Met243Val mutant, and at 412 nm for LPO, and at 428 nm for recombinant MPO. It was not possible to completely reduce the Met243 mutants at pH 7 by addition of dithionite under anaerobic conditions. Reduction was facilitated by addition of 0.5 µM methylviologen. The Soret band was found at 432 nm for Met243Cys, 447 nm for Met243Thr, first at 445 nm and finally at 430 nm for Met243Gln, and first at 447 nm and finally at 433 nm for Met243Val at the same time scale (not shown). At pH 9.3 and after addition of 0.5 µM methylviologen the Soret band was found at 432 nm, with a shoulder at 447 nm, for Met243Cys, at 447 nm for Met243Thr, at 445 nm for Met243Gln, and at 447 nm for Met243Val (Figure 1). The Soret band of the reduced state of LPO and recombinant MPO were found at 444 and 474 nm, respectively. There is a striking similarity in the behaviour of the observed bands for the reduced forms of the Met243 mutants and the known so-called...
unstable and stable reduced LPO forms, which are found at 446 and 434 nm respectively (Carlström, 1969, Paul & Ohlsson, 1985, Sievers, 1980).

Figure 1. Optical absorbance spectra of the Met243Cys, Met243Thr, Met243Gln, and Met243Val mutants, LPO, and recombinant MPO. (oxidised, solid line; reduced, dotted line) Met243 mutants samples in 100 mM sodium carbonate buffer (pH 9.3). 0.5 μM methylviologen was added prior to reduction. LPO and recombinant MPO samples in 100 mM potassium phosphate buffer (pH 7). (The spectra are normalised to give similar signal heights).
In order to investigate the type of haem present we prepared alkaline pyridine samples. Figure 2 shows that the Soret band of the different Met243 mutants is found at similar positions, but they all are blue-shifted compared to that of recombinant MPO. Mutation of the Met243 residue clearly affects the chemical nature of the haem group, and the spectrum is now similar to that of protohaem IX (Fuhrhop & Smith, 1975). Incubation of native or recombinant MPO in the pyridine solution at high pH for a period more than 5 hours resulted in a spectrum with bands at 425, 526 and 565 nm, similar to that of Met243 mutants (not shown). Newton et al. (Newton et al., 1965b) also have reported alteration of the pyridine haemochrome spectrum over a period of 6 h, with bands at 425, 526 and 565 nm. This time-dependent process may be indicative for the loss of the sulphonium ion linkage under these conditions.
Chapter 5

EPR spectra of the Met243 mutants show multiple high-spin signals, indicating the presence of multiple species in the mutants (Figure 3). Lowering the pH increased the intensity of the more rhombic species, whereas addition of chloride or addition of glycerol increased the intensity of the more axial species. Changing the buffer to Hepes (pH 7) did not affect the spectrum. Addition of cyanide to the oxidised enzyme resulted in the low-spin ferric enzyme state (Figure 4). Whereas addition of 10 mM KCN was sufficient to convert the recombinant MPO into the low-spin state \(K_d = 0.43 \mu M\) (Bolscher & Wever, 1984), up to 200 mM was needed to obtain the low-spin state of the Met243 mutants. Clearly the affinity for cyanide of the Met243 mutants is lowered. The low-spin spectrum of recombinant MPO \((g_x, g_y, g_z = 2.87, 2.25, 1.63)\) is identical to that of the native MPO \((g_x, g_y, g_z = 2.87, 2.25, 1.63)\) (not shown), and more axial than that of the low-spin spectrum of LPO \((g_x, g_y, g_z = 2.91, 2.25, 1.57)\). The low-spin species of Met243Val \((g_x, g_y, g_z = 3.03, 2.21, 1.47)\) and that of

![Figure 3. EPR spectra of the high-spin forms of Met243Cys, Met243Thr, Met243Gln, Met243Val mutant, LPO, and recombinant MPO. Met243Cys (90 μM), Met243Thr (120 μM), Met243Gln (80 μM), Met243Val (65 μM), LPO (185 μM), and recombinant MPO (30 μM). All samples in 100 mM potassium phosphate buffer (pH 7). Conditions during the recording of the spectra were as follows: temperature, 15 K; frequency, 9.41 GHz; modulation amplitude, 1.27 mT; microwave power incident to the cavity, 26 mW. (The spectra are normalised to give similar signal heights).](image-url)
Met243Gln mutant \((g_x, g_y, g_z = 3.02, 2.22, 1.48)\) have a more rhombic signal. The Met243Thr mutant differs from these in that it shows two low-spin signals \((g_x, g_y, g_z = 3.04, 2.24, 1.47\) and \(g_x, g_y, g_z = 2.90, 2.24, 1.58\)), and the latter one is similar to that observed for the Met243Cys mutant \((g_x, g_y, g_z = 2.90, 2.26, 1.58)\). As Figure 4 shows all Met243 mutants exhibit more \(g\)-strain, as indicated by the broader signals in their low-spin EPR spectra.

![EPR spectra of the low-spin forms of Met243Cys, Met243Thr, Met243Gln, Met243Val mutant MPO, LPO, and recombinant MPO. Low-spin states were obtained by addition of 70 \(\mu\)l potassium cyanide solution (0.5 M final concentration) in 100 mM sodium carbonate buffer (pH 9.5) to 200 \(\mu\)l of Met243 mutants of Figure 3, and of 5 \(\mu\)l potassium cyanide solution (10 mM final concentration) in 100 mM sodium carbonate buffer (pH 9.5) to 200 \(\mu\)l of LPO and recombinant MPO. Conditions during the recording of the spectra were as in Figure 3. (The spectra are normalised to give similar signal heights).]
Figures 5A and 5B show the resonance Raman spectra of Met243Cys, Met243Thr, Met243Gln and Met243Val mutants, LPO, and recombinant MPO. All Met243 mutants show identical spectra. Most remarkable is the effect of the Met243 mutation in the oxidation state marker region, as reported before (Kooter et al., 1997a). From the high-resolution high-frequency part of the resonance Raman spectra (Figure 5B), it is clear that the mutation results in a highly symmetric $v_4$ line at approximately 1370 cm$^{-1}$ similar in shape and position to that observed for LPO. The overall spectrum of the Met243 mutants is less complicated than that of recombinant MPO and is essentially identical to the spectrum of LPO, indicative of a haem with a higher symmetry.
Within the mammalian peroxidases family, MPO is the only peroxidase that is able to peroxidise chloride to hypochlorous acid, a bactericidal agent, at a substantial rate. Recombinant and native MPO are found to have similar kinetics parameters, as judged from the chlorination of MCD (Floris et al., 1995, Jacquet et al., 1991). We also measured the chlorination activity by means of the taurine assay, since some mutants were found to directly oxidise MCD in the presence of hydrogen peroxide, as is native MPO in the absence of a halide substrate (Harrison & Schultz, 1976, Kettle & Winterbourn, 1988). Taurine is known to be unreactive toward MPO compounds I and II (Kettle & Winterbourn, 1994, Marquez & Dunford, 1994), and in this respect taurine oxidation makes a better chlorination assay. Two classical peroxidase substrates, namely ABTS and guaiacol, were also investigated. Table 1 shows the activity of the mutants in different assays measured at pH 5 and 7 under conditions described in the 'Material and methods' section. The native MPO was shown to have its pH optimum at 7 for the guaiacol assay, whereas that for the ABTS assay was around pH 5. For the classical peroxidase substrates, a residual activity of 1% was found for the Met243Val and Met243Gln mutant, an activity of 5% was present for Met243Thr mutant, and 2% activity was present for the Met243Cys mutant. Thus, these mutations had a profound effect on the function of the
enzyme as a classical peroxidase. In the chlorination assay with taurine (pH 5), activities of less than 1.5% were found for the Met243Val and Met243Gln mutant, 15% activity was found for the Met243Thr mutant, and 1.5% activity was present for the Met243Cys mutant. In the assay using MCD at this pH 3, 6, 16 and even 56% residual activity was found for the Met243Val, Met243Gln, Met243Thr and Met243Cys mutant, respectively. However, the higher values for the chlorination activity found using this assay are probably due to direct oxidation of MCD by the mutants.

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<th>Table 1. Activity of MPO and mutants under conditions as described under Materials and methods.</th>
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*activity is expressed in s⁻¹ and was calculated from the absorbance changes and corresponding extinction coefficients.

Halides are known to interact with MPO to give spectroscopically distinguishable complexes (Bakkenist et al., 1980), and chloride shifts the Soret band in the optical absorbance spectrum from 428 nm to 434 nm (Bakkenist et al., 1980, Bolscher & Wever, 1984). At each chloride concentration, the degree of saturation can be determined from the absorbance difference between MPO and the MPO-chloride complex in the Soret region (trough at 427 and peak at 448 nm), and from the resulting saturation curve the dissociation constant (Kₐ) of the MPO-chloride complexes can be obtained. In line with the results of Bakkenist et al. (Bakkenist et al., 1980) and Bolscher and Wever (Bolscher & Wever, 1984), the Kₐ is found to be strongly pH dependent and is in the order of 8 mM at pH 5.5. Since mutation of the Met243 results in the loss of the positively charged sulphonium ion linkage, we wondered whether this would affect the chloride binding to the enzyme. Figure 6 shows the pH dependence of the dissociation constants, Kₐ's, of several mutant MPO-chloride complexes. It is evident that the dissociation constant for the Met243 mutants has increased almost 100-fold compared to the recombinant and native enzyme. For comparison, the pH dependence of the Kₐ for chloride of the Asp94Asn and Glu242Gln mutants were also measured. As seen in Figure 6, the Kₐ for chloride of the Glu242Gln mutant is of the same order of magnitude as those of the recombinant and native MPO system. For the Asp94Asn mutant a difference spectrum is observed with two difference features. Calculation of the Kₐ from the chloride-induced peak at 415 nm and the trough at 400 nm results in a Kₐ of 10 mM (X, Figure 6), whereas the calculation from the peak at 444 nm and the trough at 426 nm results in a Kₐ of 1.5 mM (●, Figure 6) at pH 4. It is known that Asp94Asn mutant consist of two species. The first one lacks the ester bond formed by Asp94 and is spectroscopically similar to native MPO, whereas the second also lacks the sulphonium ion linkage and is spectroscopically similar to a Met243 mutant (Chapter 3). The values of 1.5 and 10 mM correspond well to the first and second species.
There is still debate on the nature of the sulphonium ion linkage, and we tried to specifically investigate this by isotopically labelling of Met243 and study the Met243 labelled recombinant MPO by FTIR. For this, recombinant MPO was expressed by the CHO cell line that was grown in the presence of $^{13}$CD$_3$-labelled methionine. As a control the Met243Thr mutant was also grown in the presence of $^{13}$CD$_3$-labelled methionine. Of the nine fundamental vibrations of a methyl group, two are in the high frequency region (3000 cm$^{-1}$). An asymmetrical stretching mode, $v_4$, is found at 2962 cm$^{-1}$ and a symmetrical stretching mode, $v_2$, at 2872 cm$^{-1}$ (Bellamy, 1956). In case of $^{-13}$CD$_3$ (in stead of -CH$_3$) we should expect these bands to appear in the 2200 cm$^{-1}$ region. Indeed for $^{-13}$CD$_3$-methionine in solution two bands are observed in the 2300-2000 cm$^{-1}$ wavelength region (Figure 7A). The band at 2230 cm$^{-1}$ is assigned as the asymmetric stretch ($v_4$), and the band at 2129 cm$^{-1}$ as the symmetric stretch group frequency ($v_2$) of $^{-13}$CD$_3$ (Meloan, 1963). In the solid state form of $^{13}$CD$_3$-methionine, the $v_4$ mode down shifts 13 cm$^{-1}$, to 2217 cm$^{-1}$, whereas the $v_2$ mode down shifts 11 cm$^{-1}$, to 2128 cm$^{-1}$, compared to the solution state (Figure 7B). Methylation of the $^{13}$CD$_3$-methionine results in a sulphonium ion structure (Toennies & Kolb, 1945). In this methylated $^{13}$CD$_3$-methionine in solution, figure 7C, $v_4$ is found now at 2259 cm$^{-1}$ and the $v_2$ mode at 2134 cm$^{-1}$. The up shift of 29 cm$^{-1}$ of the $v_4$ band is large, compared to the shift of the $v_2$ band of 5 cm$^{-1}$. However it is known that the position of $v_4$ is affected most by its surroundings. Also the relative intensities of the $v_4$ and $v_2$ bands invert. It is known that the relative intensity of the asymmetrical stretching mode of a methyl group is much greater in compounds with a higher proportion of branched chains (Bellamy, 1956), as is the case in the
methylated $^{13}$CD$_3$-methionine. Going from solution to solid state for the methylated $^{13}$CD$_3$-methionine results in down shifts of 21 cm$^{-1}$ for $v_4$ and 18 cm$^{-1}$ for $v_1$ (Figure 7D).

Recombinant MPO contains 22 methionine residues, of which only the Met243 is of interest for this study. In an absolute FTIR spectrum it would be impossible to identify specific bands corresponding to Met residues, due to the high background absorbance. Therefore reduced-oxidised spectra of a labelled recombinant MPO are recorded. In a previous FTIR study this detection method has allowed us to identify specific ester bonds sensitive to the oxidation state of the enzyme (Kooter et al., 1997b). Figure 8A shows such a difference spectrum of recombinant MPO that is produced in the presence of $^{13}$CD$_3$-methionine. It is clear that distinct negative and positive bands are observed, corresponding in position to those seen for methylated-$^{13}$CD$_3$-methionine. However, a positive identification is still lacking and therefore also the reduced-oxidised FTIR spectrum of the Met243Thr mutant, produced in the presence of $^{13}$CD$_3$-labelled methionine, was recorded. Indeed Figure 8B shows the bands observed originally in spectrum 8A have disappeared for the main part. The difference spectrum of trace A and B should correspond to the contribution of Met243 only. Figure 8C shows two positive bands at 2236 and 2119 cm$^{-1}$, originating from the reduced enzyme state, and two negative bands, at 2227 and 2112 cm$^{-1}$, originating from the oxidised enzyme state. The $v_4$ band seems to be slightly more affected by the oxidation state of the enzyme than the $v_1$ band, being shifted by 9 cm$^{-1}$ compared to 7 cm$^{-1}$. The positions of the positive bands are almost identical to those of the solid state form of the methylated methionine model compound.
Discussion

In a recent study (Kooter et al., 1997a), we have already shown that the non-conserved residue in the mammalian peroxidase family Met243 is responsible for the unique characteristics of MPO. By mutation of this residue into a glutamine, an enzyme resulted with properties similar to LPO. We have now also mutated the Met243 into residues found in the other mammalian peroxidases (threonine for EPO and a valine for TPO).

Mutation of Met243 of MPO results in a blue shift of the Soret band to a position similar to that found for the other mammalian peroxidases. As observed before (Kooter et al., 1997a), it is difficult to reduce the Met243 mutants but addition of the mediator methyl viologen facilitates the reduction. At pH 7 this results in a Soret band found first at approximately 446 nm and later at approximately 432 nm for Met243Gln and Met243Val mutant, whereas for Met243Thr mutant it remains at 447 nm and for Met243Cys mutant it is found immediately at 432 nm. At pH 9.3 for all mutants the Soret band is found at approximately 447 nm, except the Met243Cys mutant, which has its main band at 432 nm and a shoulder at 447 nm. LPO displays two spectrally different ferrous forms upon reduction (Carlström, 1969; Sievers, 1980). The initially unstable form (Soret band at 446 nm) spontaneously
converts into a stable one (Soret band at 435 nm), especially at low pH (Ohlsson, 1984); this was suggested to be caused by changes in the protein conformation (Carlström, 1969, Sharonov, 1996). A carboxyl group (pKₐ 3-3.5) was suggested to be involved in this conversion (Ohlsson, 1984), and a carboxylate has also been previously suggested as the sixth ligand of the haem iron at room temperature (Sievers et al., 1983). The conversion of the Soret bands from 446 nm to 432 nm indicates that similar processes as in LPO, occur for the Met243 mutants.

Taylor et al. (Taylor et al., 1995) state that the contribution of the electrophilic sulphonium ion linkage to the red shift of the Soret maximum of the native oxidised enzyme would be small. We have shown that this is not the case, when this linkage is broken through mutation of Met243 a blue shift of up to 18 nm occurs, which is even larger in the reduced state.

It is obvious from the pyridine haemochrome spectra that mutation of the methionine residue affected the chemical nature of the haem group present in MPO. The Soret band in the pyridine haemochrome spectrum of the Met243 mutant is blue-shifted by 19 nm to approximately 419 nm, and is similar in position to that of protohaem IX (Fuhrhop & Smith, 1975) and comparable to that of LPO. It is clear that the Met243 mutants, which all lack the sulphonium linkage, have similar positions of their Soret and α bands. Prolonged incubation of the native or recombinant MPO in alkaline pyridine resulted in a shift of the Soret band towards 420 nm, as reported before (Newton et al., 1965b). Since the resulting spectrum is similar to that of a Met243 mutant, this may indicate the loss of the sulphonium ion linkage under these conditions. Similar shifts in the pyridine haemochrome spectra were observed when MPO was incubated with borohydride, hydrazine and bisulphite. This was taken as proof for the presence of an aldehyde group (Harrison & Schultz, 1978). The presence of the sulphonium ion linkage, however, offers a reasonable explanation for the reactivity with these carbonyl reagents (Taylor et al., 1995).

The EPR high-spin spectra are indicative of inhomogeneous mutant species. For this reason we also recorded the low-spin form of the mutants, formed by addition of cyanide. The low-spin enzyme state of the Met243Val, Met243Gln and Met243Thr mutant shows a single EPR signal that is more rhombic than that of recombinant or native MPO. The Met243Thr mutant also shows a second signal, which is similar to that of the Met243Cys mutant. It is not clear why two cyanide derivatives in the Met243Thr mutant are present, or how the second derivative is related to the species of Met243Cys mutant MPO. Since addition of a smaller volume of a pH 7 buffered potassium cyanide solution to the Met243Thr mutant MCD sample resulted only in the formation of the more axial species (Chapter 6), the origin of the two species may be due to a pH effect. All Met243 mutants show g-strain in their low-spin EPR spectra, indicated by the broader signals. This originates from a slightly different conformation of the iron site of each protein, resulting in a distribution of g-values (Fritz et al., 1971). Thus, the EPR data of Met243 mutants are indicative of more internal flexibility and microheterogeneity of the haem iron in the protein. Mutation of the neighbouring residue, Glu242, also shows g-strain in its low-spin EPR spectrum (Chapter 3).

The resonance Raman spectrum of MPO is rather complex, especially in the oxidation-state marker (ν₄) region (1367 cm⁻¹), where multiple lines arise due to the symmetry reduction of the haem group suggesting that the prosthetic group of MPO had a relatively low symmetry. Mutation of Met243 has a drastic effect on the resonance Raman spectrum. In the oxidation-state marker region (ν₄) a singlet line is now observed at approximately 1371 cm⁻¹. In the Raman spectrum of the Met243 mutants, Raman bands with
A 16 symmetry (1563, 1485, 1367, 675 cm⁻¹, values of Met243Gln mutant MPO) have the highest intensities, suggesting a chromophore structure comparable to that of LPO with a symmetry close to D₄h. In the recombinant MPO the B₁₂ (1614, 1551, 1379, 717 cm⁻¹), A₂ (1307 cm⁻¹) and B₂ (1394 cm⁻¹) modes become relatively more enhanced compared to the A₁₂ modes, as a result of symmetry reduction. Mutation of Glu242 results also in resonance Raman spectra that are indicative of a haem group with a higher symmetry than found for native or recombinant MPO (Chapter 3) (Floris et al., 1995). Loss of either the sulphonium ion linkage or the Glu242 ester bond therefore results in a more symmetric haem group in these mutants of MPO.

Mutation of Met243 has a huge effect on the activity of the enzyme. Except for the Met243Thr mutant, none of the Met243 mutants shows chlorination activity. In EPO a threonine is present at this position instead of a methionine, and it has been reported that EPO is also able to carry out the peroxidative chlorination of monochlorodimedon, although the kinetic properties differ (Buys et al., 1984, Wever et al., 1981). In this respect it is interesting that also the Met243Thr mutant still has some chlorinating activity. At pH 5, a 15% residual chlorinating activity as measured via the taurine assay is found for Met243Thr compared to the recombinant MPO, whereas the other Met243 mutants only show about 1.5% residual activity. For the Met243Cys mutant a considerable activity is found in the MCD assay. However, using the taurine assay only a very low activity is detected. This indicates that the Met243Cys mutant is probably capable of direct oxidation of MCD. In the classical peroxidase assays about 5% residual activity is found for the Met243Cys and Met243Thr mutants at the pH optimum for the native system (which is at pH 7 for guaiacol and at pH 5 for ABTS). For the Met243Gln and Met243Val mutants only 1% residual activity is found for these conditions. It is interesting to note that the chlorinating activity of native and recombinant enzyme as measured at pH 7.0 by the taurine assay is much higher than using the MCD assay. This is in line with the results by Kettle and Winterbourn (Kettle & Winterbourn, 1994), who found at pH 7.4 an approximately 20-fold higher chlorinating activity for the taurine assay compared to the MCD assay. This diminished activity might be due to the reaction of MCD with compound I, trapping MPO as compound II, which is inactive in the chlorination reaction (Bolscher et al., 1984b, Kettle & Winterbourn, 1988).

Since mutation of Met243 results in the loss of the positively charged sulphonium ion linkage, we investigated the effect on the binding properties of the negatively charged chloride ion for the different Met243 mutants. The dissociation constant, K_d, for chloride is strongly pH dependent and increases almost 100-fold upon mutation of Met243. This increase seems to be solely due to the loss of the positive charge and not to any conformational changes, since mutation of Glu242, responsible for the neighbouring ester bond, or the Asp94 responsible for the other ester bond, does not affect the dissociation constant, K_d, for chloride. The EPR data show that also the affinity for cyanide of the Met243 mutants is lowered considerably.

With help of difference FTIR spectroscopy we have now been able to detect a single methionine in a protein. The positions of the 13CD₃-stretches observed in the difference FTIR spectrum of recombinant MPO grown on 13CD₃-labelled methionine correspond well with the positions found in the methylated 13CD₃-methionine model compound. More model compound studies are required to see if we are able to distinguish between the two models proposed for the sulphonium ion linkage. In one model there is a sulphonium ion linkage to the haem via an intact vinyl group (Figure 9A) (Fenna et al., 1995, Taylor et al., 1995), whereas in
analogy with the binding of the haem in cytochrome c, we recently proposed a model where the α-carbon of the vinyl group is involved (Figure 9B) (Kooter et al., 1997b). It is clear from our results that difference FTIR may become a powerful technique in specific detection of isotopically labelled single residues, in particular when combined with site-directed mutagenesis studies.

There are no indications that in the Met243Cys mutant the Cys243 residue forms a covalent linkage to the haem group. Although in such a linkage a positive charge is absent at the sulphur atom, as in haem c, a linkage should put the haem group in a fixed position with a lower symmetry, resulting in a more complicated resonance Raman spectrum and in a low-spin EPR spectrum with less g-strain.

In the past spectroscopic and chemical evidence for the presence of a formyl-containing haem in MPO has been presented (Harrison & Schultz, 1978, Odajima, 1980, Schultz & Shmukler, 1964, Sono et al., 1991). In terms of the present knowledge this can now be explained in the following way. First, both a formyl substituent on the haem periphery as well as a sulphonium ion linkage act as a electron-withdrawing group. Thus, the spectral properties of the pyridine haemochrome and the inverse sign pattern of the Soret band found in the MCD spectrum can also be explained by the electron withdrawing properties of the sulphonium ion linkage. Secondly, as already mentioned by Taylor et al. (Taylor et al., 1995), the presence of the methylion sulphonium ion linkage may offer a reasonable explanation for the reactivity of MPO with carbonyl reagents such as borohydride and hydroxylamine (Harrison & Schultz, 1978, Odajima, 1980). It is known that this type of covalent linkage is cleaved under reducing conditions (Naider & Bohak, 1972). Spectroscopic evidence for a chlorin-like haem structure came from resonance Raman (Babcock et al., 1985, Sibbett & Hurst, 1984) and early MCD spectra (Eglinton et al., 1982, Sono et al., 1986). The neighbouring residues Glu242 and Met243 cause considerable distortion from the planar conformation, resulting in a lower symmetry as indicated by the resonance Raman spectrum of MPO. Mutation of Met243 resulted in a normal LPO-like MCD spectrum (Chapter 6). Whether this is purely due to the removal of the electron-withdrawing character of the sulphonium ion linkage or also to symmetry reduction of the haem may be checked by studying the MCD of the Glu242 mutant.

The remaining questions still concern the exact structure of the sulphonium ion linkage and more importantly how it is formed. Based on mass spectrometry of a haem group obtained by autolytic cleavage and proteolytic digestions Taylor et al. (Taylor et al., 1995) proposed a model for the haem group of MPO as in figure 9A. Based on analogy to the chemistry involved in formation of the thioether groups that are present in cytochrome c, we suggested, as seen in figure 9B (Kooter et al., 1997b), the presence of a bond between the methionine sulphur atom and the α-carbon of the vinyl group rather than an unprecedented vinyl sulphonium ion. An extended Beilstein search for methionine sulphonium structures with an attached vinyl group, similar as predicted by Taylor et al. (Taylor et al., 1995), resulted in no matches, which might be indicative of the improbability of this structure. Future vibrational spectroscopy experiments on isotopically labelled residues might resolve the question of the exact chemical nature of the sulphonium ion linkage.
Sulphonium ion linkage

Figure 9. Proposed haem structures of MPO. (A) by (Fenna et al., 1995, Taylor et al., 1995) (B) by (Kooter et al., 1997b).

The second question is, whether an enzyme for the formation of this special methionine sulphonium ion linkage is required in MPO, or that it is formed autocatalytically, as has been proposed for the two ester linkages of the haem group the Asp94 and Glu242 residue (DeFitts et al., 1997). The fact that active MPO can only be expressed by a mammalian cell line, like CHO, might suggest that production of this enzyme requires some additional cofactors present in higher organisms.

In conclusion, we may say that mutation of Met243 results in a mutant MPO that has similar characteristics to the other mammalian peroxidases. Two different effects of this mutation can be distinguished, the first one is due to the loss of the electron-withdrawing positive charge (which affects chloride and cyanide binding properties) and the second is the loss of the bowed shape and distortion from the planar conformation of the haem group, resulting in a lower symmetry, as evidenced by less complicated resonance Raman spectra. The latter effect can also be accomplished by mutation of the neighbouring Glu242 residue (Chapter 3; Floris et al., 1995). It is the combination of these two neighbouring covalent linkages which induces the asymmetry in the haem macrocycle and may place the haem group in the right spatial configuration.
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Chapter 6

The 'sulphonium ion linkage is responsible for the anomalous magnetic circular dichroism and optical spectral properties of myeloperoxidase.
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