The Sulfonium Ion Linkage in Myeloperoxidase

DIRECT SPECTROSCOPIC DETECTION BY ISOTOPIC LABELING AND EFFECT OF MUTATION*

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The heme group of myeloperoxidase is covalently linked via two ester bonds to the protein and a unique sulfonium ion linkage involving Met243. Mutation of Met243 into Thr, Gln, and Val, which are the corresponding residues of eosinophil peroxidase, lactoperoxidase, and thyroid peroxidase, respectively, and into Cys was performed. The Soret band in the optical absorbance spectrum in the oxidized mutants is now found at approximately 411 nm. Both the pyridine hemochrome spectra and resonance Raman spectra of the mutants are affected by the mutation. In the Met243 mutants the affinity for chloride has decreased 100-fold. All mutants have lost their chlorination activity, except for the M243T mutant, which still has 15% activity left. By Fourier transform infrared difference spectroscopy it was possible to specifically detect the 13CD3-labeled methionyl sulfonium ion linkage. We conclude that the sulfonium ion linkage serves two roles. First, it serves as an electron-withdrawing substituent via its positive charge, and, second, together with its neighboring residue Glu242, it appears to be responsible for the lower symmetry of the heme group and distortion from the planar conformation normally seen in heme-containing proteins.

In the family of mammalian peroxidases, myeloperoxidase (MPO) is an extraordinary peroxidase. First of all, the enzyme is the only mammalian peroxidase known to peroxidize chloride to hypochlorous acid at a substantial rate. Secondly, MPO differs in its spectroscopic characteristics by its unusual redshifted Soret band in the optical absorbance as well as in its pyridine hemochrome spectrum, its complicated resonance Raman spectrum, and its inverted sign pattern of the Soret band in the MCD spectrum (1–6). Those differences have been attributed to the special nature or structure of the heme group in MPO. Because this heme is covalently bound to the protein, characterization of this chromophore has been difficult. Based on different spectroscopic techniques, a formyl-containing heme α, a chlorin, and a heme b prosthetic group have been proposed in the past (3, 5, 7–9). 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) (10–12), 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 (13). The structural data for human MPO suggested that three heme 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 heme groups in all mammalian peroxidases via the conserved aspartate and glutamate residues (14). The third linkage involves the nonconserved Met243 residue, for which there is considerable evidence. Based on the unique autoleavage 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. (15) proposed that Met243 was involved in a sulfonium ion linkage to the heme group. Later studies showed that this sulfonium ion linkage involved the vinyl group of pyrrole ring A (13, 16).

Met243 is replaced by a threonine in human EPO (10), whereas in bovine LPO a glutamine is found at this position (17), and, as recently shown by Ueda et al. (11), 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 (18). In a recent study (19), we have mutated the Met243 of MPO into a glutamine 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 to investigate whether this residue would still be able to make a linkage to the heme group via its sulfur atom, in analogy with heme c. These mutations of the Met243 residue in MPO resulted in loss of the typical MPO enzymatic and spec-
trosopic characteristics. The binding of chloride to the Met\textsuperscript{243} mutants is also affected; the affinity for Cl\textsuperscript{-} 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 labeling of the recombinant wild type and mutant MPO.

**EXPERIMENTAL PROCEDURES**

Transfection of recombinant plasmids into Chinese hamster ovary (CHO) cells, selection and culture procedures for transfected cells, protein purification protocols, Western blotting, enzyme-linked immunosorbent assay, and electrophoretic analysis of recombinant myeloperoxidase were described in detail previously (20). The M243C, M243T, M243Q, and M243V mutant proteins were produced by replacing, in the myeloperoxidase-coding cassette carried by plasmid pNIV2703, a 178-base pair ApaI-AuII 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 liters) was collected, and the mutant was purified (20). The \textsuperscript{13}C\textsubscript{3}-labeled methionine recombinant and M243T mutant were produced similarly to the ordinary recombinant and M243T mutant, but the CHO cells were grown in the presence of 1 gram of \textsuperscript{13}C\textsubscript{3}-labeled methionine (Isosect Inc.).

It was found that the recombinant MPO had a lower \( R_z \) value 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\textsuperscript{-1}cm\textsuperscript{-1} of native MPO. Recombinant MPO has a lower \( R_z \) value (0.6) than natural MPO (0.8) because the 84-kDa recombinant MPO is produced as a single chain monomer and is secreted as the covalently linked dimer (24)). The \( R_z \) of the recMPO is lower compared with that of the natural enzyme because of a higher content of phenylalanine and tyrosine residues in the unprocessed recMPO. However, the recMPO is very similar if not identical in specific activity and spectral properties as detailed in Ref. 20.

All optical spectra for the recombinant and mutants were recorded on a Cary 50 Biopec spectrophotometer. An appropriate amount of dithionite solution was added for reduction. The pyridine hemochrome spectra were prepared in 2.1M pyridine and 75 mM NaOH, and a concentrated nitrite solution was used for reduction. The pyridine hemochrome spectra were identical in specific activity and spectral properties as detailed in Ref. 20.

**RESULTS**

Here we present a spectroscopic and enzymatic characterization of all Met\textsuperscript{243} mutants available; for comparison, LPO data are also shown. Fig. 1 shows the optical absorbance spectra of both the oxidized and reduced forms of the M243C, M243T, M243Q, and M243V mutants, LPO, and recombinant MPO. The Soret band in the oxidized enzyme state was found at 410 nm for M243C, 414 nm for M243T, 410 nm for M243Q,
414 nm for M243V mutant, 412 nm for LPO, and 428 nm for recombinant MPO. At pH 7 it was not possible to completely reduce the Met243 mutants by addition of dithionite under anaerobic conditions. Reduction was facilitated by addition of 0.5 mM methylviologen. The Soret band was found at 432 nm for M243C, 447 nm for M243T, first at 445 nm and finally at 430 nm for M243Q, and first at 447 nm and finally at 433 nm for M243V at the same time scale (not shown). At pH 9.3 and after addition of 0.5 mM methylviologen reduction of the Met243 mutants is complete and the Soret band was found at 432 nm, with a shoulder at 447 nm, for M243C, at 447 nm for M243T, at 445 nm for M243Q, and at 447 nm for M243V (Fig. 1). The Soret band of the reduced state of LPO and recombinant MPO at pH 7 were found at 444 and 474 nm, respectively.

To investigate the type of heme present we prepared alkaline pyridine samples. Fig. 2 shows that the Soret band of the different Met243 mutants is found at similar positions, but they all are blue-shifted compared with that of recombinant MPO. Mutation of the Met243 residue clearly affects the chemical nature of the heme group, and the spectrum is now similar to that of protoheme IX (29). Incubation of native or recombinant MPO in the pyridine solution at high pH for a period more than 5 h resulted in a spectrum with bands at 425, 526, and 565 nm, similar to that of Met243 mutants (not shown).

EPR spectra of the Met243 mutants show multiple high spin signals, indicating the presence of multiple species in the mutants (Fig. 3). Lowering the pH increased the intensity of the more rhombic species, whereas addition of chloride or addition of glyceral 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 oxidized enzyme resulted in the low spin ferric enzyme state (Fig. 4). Whereas addition of 10 mM KCN was sufficient to convert the recombinant MPO into the low spin state ($K_d = 0.43 \mu M$ (30)), 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 ($g_1, g_2, g_3 = 2.87, 2.25, 1.63$) is identical to that of the native MPO ($g_1, g_2, g_3 = 2.87, 2.25, 1.63$) (not shown) and more axial than that of the low spin spectrum of LPO ($g_1, g_2, g_3 = 2.91, 2.25, 1.57$). The low spin species of M243V ($g_1, g_2, g_3 = 3.03, 2.21, 1.47$) and that of M243Q mutant ($g_1, g_2, g_3 = 3.02, 2.22, 1.48$) have a more rhombic signal. The M243T mutant differs from these in that it shows two low spin signals ($g_1, g_2, g_3 = 3.04, 2.24, 1.47$ and $g_1, g_2, g_3 = 2.90, 2.24, 1.59$), and the latter one is similar to that observed for the M243C mutant ($g_1, g_2, g_3 = 2.90, 2.26, 1.58$). As Fig. 4 shows, all Met243 mutants exhibit more $g$ strain, as indicated by the broader signals in their low spin EPR spectra.

Fig. 5 shows the resonance Raman spectra of M243C, M243T, M243Q, and M243V 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 (19). It is clear that the
mutation results in a highly symmetric ν₄ 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 heme with a higher symmetry.

Within the mammalian peroxidases family, MPO is the only peroxidase that is able to peroxidize 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 (31, 32). We also measured the chlorination activity by means of the taurine assay, because some mutants were found to directly oxidize MCD in the presence of hydrogen peroxide, as is native MPO in the absence of a halide substrate (33, 34). Taurine is known to be unreactive toward MPO compounds I and II (35, 36), and in this respect taurine oxidation makes a better chlorination assay. Two classical peroxidase substrates, namely ABTS and guaiacol, were also investigated. Table I shows the activity of the mutants in different assays measured at pH 5 and 7 under conditions described under “Experimental Procedures.” 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 M243V and M243Q mutant, an activity of 5% was present for M243T mutant, and 2% activity was present for the M243C 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 M243V and M243Q mutant, 15% activity was found for the M243T mutant, and 1.5% activity was present for the M243C mutant. In the assay using MCD at this pH, 3, 6, 16 and even 56% residual activity was found for the M243V, M243Q, M243T, and M243C 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.

Halides are known to interact with MPO to give spectroscopically distinguishable complexes (28, 37), and chloride shifts the Soret band in the optical absorbance spectrum from 428 to 434 nm (28, 30). 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 nm and peak at 448 nm), and from the resulting saturation curve the dissociation constant (Kₒ) of the MPO-chloride complexes can be obtained. In line with the results of Bakkenist et al. (28) and Bolscher and Wever (30), the Kₒ is found to be strongly pH-dependent and is in the order of 8 mM at pH 5.5. Because mutation of the Met²⁴³ results in the loss of the positively charged sulfonium ion linkage, we won-
In this methylated 13CD3-methionine in solution (Fig. 7), of 13CD3-labeled methionine. As a control the M243T mutant expressed by the CHO cell line that was grown in the presence of 13CD3-methionine, the relative intensities of the bands are observed in the 2300–2000 cm⁻¹ region. In the solid state that is produced in the presence of 13CD3-methionine results in downshifts of 21 cm⁻¹ for 13CD3 (39). In the solution that is produced in the presence of 13CD3-methionine results in downshifts of 21 cm⁻¹ for 13CD3 (39). The nine fundamental vibrations of a methyl group, two are in the high frequency region (3000 cm⁻¹). An asymmetrical stretching mode, ν₁, is found at 2963 cm⁻¹ and a symmetrical stretching mode, ν₂ at 2872 cm⁻¹ (38). In case of 13CD₃ (instead of CH₃) we should expect these bands to appear in the 2200 cm⁻¹ region. Indeed for 13CD₃-methionine in solution two bands are observed in the 2300–2000 cm⁻¹ wavelength region (Fig. 7A). The band at 2230 cm⁻¹ is assigned as the asymmetric stretch (ν₁), and the band at 2129 cm⁻¹ as the symmetric stretch group frequency (ν₂) of 13CD₃ (39). In the solid state form of 13CD₃-methionine, the ν₂ mode downshifts 13 cm⁻¹ to 2217 cm⁻¹, whereas the ν₁ mode downshifts 11 cm⁻¹ to 2128 cm⁻¹, compared with the solution state (Fig. 7B). Methylation of the 13CD₃-methionine results in a sulfonium ion structure (21). In this methylated 13CD₃-methionine in solution (Fig. 7C), ν₁ is found now at 2259 cm⁻¹ and the ν₂ mode at 2134 cm⁻¹. The up shift of 29 cm⁻¹ of the ν₂ band is large, compared with the shift of the ν₁ band of 5 cm⁻¹. However, it is known that the position of ν₂ is affected most by its surroundings. Also the relative intensities of the ν₁ and ν₂ 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 (38), as is the case in the methylated 13CD₃-methionine. Going from solution to solid state for the methylated 13CD₃-methionine results in downshifts of 21 cm⁻¹ for ν₂ and 18 cm⁻¹ for ν₁ (Fig. 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, because of the high background absorbance. Therefore reduced oxidized spectra of a labeled 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 (14). Fig. 8A shows such a difference spectrum of recombinant MPO in solution that is produced in the presence of 13CD₃-methionine. It is clear that distinct negative and positive bands are observed, corresponding in position to those seen for methylated 13CD₃-methionine. However, a positive identification is still lacking, and therefore also the reduced-oxidized FTIR spectrum of the M243T mutant, produced in the presence of

| Table I Activity of MPO and mutants under conditions as described under “Experimental Procedures” |
|------------------------------------------------------|
|                     | Gaussia | ABTS    | MCD     | Taurine |
|----------------------|---------|---------|---------|---------|
|                      | pH 7    | pH 5    | pH 7    | pH 5    | pH 7    | pH 5    | pH 7    | pH 5    |
| wtMPO                | 99      | 26      | 2.9     | 128     | 0.6     | 7.1     | 6.7     | 5.9     |
| recMPO               | 120     | 34      | 0.5     | 129     | 0.7     | 7       | 4.6     | 6.6     |
| M243C                | 2.3     | 0.34    | 2.4     | 7.3     | 0.1     | 3.9     | <0.1    | 0.1     |
| M243T                | 6.5     | 1.0     | 2.4     | 6.7     | 0.1     | 1.1     | 0.3     | 1.0     |
| M243Q                | 0.9     | 0.17    | 0.28    | 1.2     | ND      | 0.1     | <0.1    | <0.1    |
| M243V                | 1.3     | 0.10    | 0.10    | 1.3     | ND      | 0.2     | ND      | ND      |

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6 Kooter, I. M., Moguilovsky, N., Bollen, A., Sijtsema, N. M., Otto, C., Dekker, H. L., and Wever, R. (1999) Eur. J. Biochem. 263, 1–8.
Mutation of Met 243 of MPO results in a blue shift of the Soret band to a position similar to that found for the other mammalian peroxidase family members (16). It should be noted that also photochemically modified myeloperoxidase has spectra similar to those of the Met243 mutants (43).

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 M243V, M243Q, and M243T mutants shows a single EPR signal that is more rhombic than that of recombinant or native MPO. The M243T mutant also shows a second signal, which is similar to that of the M243C mutant. It is not clear why two cyanide derivatives in the M243T mutant are present or how the second derivative is related to the species of M243C mutant MPO. All Met243 mutants show spectra similar to those of the Met243 mutants (49).

DISCUSSION

In a recent study (19), we have already shown that the nonconserved 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 with properties similar to those of LPO. We have now also mutated the Met243 into residues found in 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 in both the oxidized and reduced state. There is also a striking similarity in the behavior 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 (40–42).

Taylor et al. (16) state that the contribution of the electrophilic sulfonium ion linkage to the red shift of the Soret maximum of the native oxidized 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 hemochrome spectra that mutation of the methionine residue affected the chemical nature of the heme group present in MPO. The Soret band in the pyridine hemochrome spectrum of the Met243 mutant is blue-shifted by 19 nm to approximately 419 nm and is similar in position to that of protoheme IX (29) and comparable with that of LPO. It is clear that the Met243 mutants, which all lack the sulfonium linkage, have similar positions of their Soret and \( \alpha \) bands. Prolonged incubation of the native or recombinant MPO in alkaline pyridine resulted in a shift of the Soret band toward 420 nm, as reported before (2). Because the resulting spectrum is similar to that of a Met243 mutant, this may indicate the loss of the sulfonium ion linkage under these conditions. Similar shifts in the pyridine hemochrome spectra were observed when MPO was incubated with borohydride, hydrazine, and bisulfite. This was taken as proof for the presence of an aldehyde group (8). The presence of the sulfonium ion linkage, however, offers a reasonable explanation for the reactivity with these carbonyl reagents (16). It should be noted that also photochemically modified myeloperoxidase has spectra similar to those of the Met243 mutants (49).

\[ \text{FIG. 7. FTIR spectra of } ^{13}\text{CD}_3\text{-labeled methionine and its methylated form, both in solution and solid state. A, } ^{13}\text{CD}_3\text{-labeled methionine solution. B, } ^{13}\text{CD}_3\text{-labeled methionine solid state. C, methylated } ^{13}\text{CD}_3\text{-labeled methionine solution. D, methylated } ^{13}\text{CD}_3\text{-labeled methionine solid state.} \]

\[ \text{FIG. 8. Reduced-oxidized FTIR difference spectra of } ^{13}\text{CD}_3\text{-methionine-labeled recombinant MPO and } ^{13}\text{CD}_3\text{-methionine-labeled M243T mutant. A, } ^{13}\text{CD}_3\text{-methionine-labeled recombinant MPO (1.4 mM). B, } ^{13}\text{CD}_3\text{-methionine-labeled M243T mutant (1.5 mM). C, difference spectrum of } ^{13}\text{CD}_3\text{-methionine-labeled recombinant MPO minus } ^{13}\text{CD}_3\text{-methionine-labeled M243T mutant. The solution samples were in 50 mM potassium phosphate buffer with 25 mM EDTA and 2.5 mM deazaflavin (pH 7.0). Each spectrum is the sum of 762 scans, with 2 cm}^{-1} \text{ resolution.} \]
tion of the neighboring residue, Glu\textsuperscript{242}, also shows a broadened low spin EPR signal.\textsuperscript{2}

The resonance Raman spectrum of MPO is rather complex, especially in the oxidation state marker (ν\textsubscript{s}) region (1367 cm\textsuperscript{-1}), where multiple lines arise because of the symmetry reduction of the heme group, suggesting that the prosthetic group of MPO had a relatively low symmetry. Mutation of Met\textsuperscript{243} has a drastic effect on the resonance Raman spectrum. In the oxidation state marker region (ν\textsubscript{s}) a singlet line is now observed at approximately 1371 cm\textsuperscript{-1}. In the Raman spectrum of the Met\textsuperscript{243} mutants, Raman bands with A\textsubscript{1g} symmetry (1563, 1485, 1367, and 675 cm\textsuperscript{-1}, values of M243Q mutant MPO) have the highest intensities, suggesting a chromophore structure comparable with that of LPO with a symmetry close to D\textsubscript{4h}. In the recombinant MPO the B\textsubscript{1g} (1614, 1551, 1379, and 717 cm\textsuperscript{-1}), A\textsubscript{2g} (1307 cm\textsuperscript{-1}), and B\textsubscript{2g} (1394 cm\textsuperscript{-1}) modes become relatively more enhanced compared with the A\textsubscript{1g} modes, as a result of symmetry reduction. Mutation of Glu\textsuperscript{242} results also in resonance Raman spectra that are indicative of a heme group with a higher symmetry than found for native or recombinant MPO (32). Loss of either the sulfonium ion linkage or the Glu\textsuperscript{242} ester bond therefore results in a more symmetric heme group in these mutants of MPO.

Mutation of Met\textsuperscript{243} has a huge effect on the activity of the enzyme. Except for the M243T mutant, none of the Met\textsuperscript{243} 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 (45, 46). In this respect it is interesting that the M243T mutant also still has some chlorinating activity. 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 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 (34, 47).

Because mutation of Met\textsuperscript{243} results in the loss of the positively charged sulfonium ion linkage, we investigated the effect on the binding properties of the negatively charged chloride ion for the different Met\textsuperscript{243} mutants. The dissociation constant, K\textsubscript{d} for chloride is strongly pH-dependent and increases almost 100-fold upon mutation of Met\textsuperscript{243}. This increase seems to be solely due to the loss of the positive charge and not to any conformational changes, because mutation of Glu\textsuperscript{242}, responsible for the neighboring ester bond, or the Asp\textsuperscript{94} responsible for the other ester bond, does not affect the dissociation constant, K\textsubscript{d} for chloride. The available data on the pH dependence of the apparent dissociation constant (Fig. 6) for the mutant chloride complexes do not allow us to quantitatively discuss whether the intrinsic dissociation constant for chloride binding, the pK\textsubscript{a} of the group involved, or both are affected in the Met\textsuperscript{243} mutants. The EPR data show that the affinity for cyanide of the Met\textsuperscript{243} mutants is also lowered considerably.

There are no indications that in the M243C mutant the Cys\textsuperscript{243} residue forms a covalent linkage to the heme group. Although in such a linkage a positive charge is absent at the sulfur atom, as in heme c, a linkage should put the heme 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 heme in MPO has been presented (7, 8, 49, 50). In terms of the present knowledge this can now be explained in the following way. First, both a formyl substituent on the heme periphery as well as a sulfonium ion linkage act as an electron-withdrawing group. Thus, the spectral properties of
The pyridine hemochrome and the inverted sign pattern of the Soret band found in the MCD spectrum can also be explained by the electron withdrawing properties of the sulfonium ion linkage. Secondly, as already mentioned by Taylor et al. (16), the presence of the methionyl sulfonium ion linkage may offer a reasonable explanation for the reactivity of MPO with carbonyl reagents such as borohydride and hydroxylamine (8, 49). It is now known that this type of covalent linkage is closed under reducing conditions (51). Spectroscopic evidence for a chlorin-like heme structure came from resonance Raman (3, 4) and early MCD spectra (5, 6). The neighboring 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 (19, 32). Mutation of Met243 resulted in a normal LPO-like MCD spectrum.3 Whether this is purely due to the removal of the electron-withdrawing character of the sulfonium ion linkage or also to symmetry reduction of the heme may be checked by studying the MCD of the Glu242 mutant.

The remaining questions still concern the exact structure of the sulfonium ion linkage and more importantly how it is formed. Based on mass spectrometry of a heme group obtained by autolytic cleavage and proteolytic digestions, Taylor et al. (16) proposed a model for the heme group of MPO as in Fig. 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 Fig. 9B (14), 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. An extended Beilstein search for methionine sulfonium structures with an attached vinyl group, similar to that predicted by Taylor et al. (16), resulted in no matches, which might be indicative of the improbability of this structure. With the help of difference FTIR spectroscopy, we have now been able to detect this methionine residue in myeloperoxidase. The positions of the 13CD33 and 15CD34 observed in the difference FTIR spectrum of recombinant MPO grown on 13CD3-labeled methionine correspond well with the positions found in the methylated 13CD3-methionine model compound. More compound models are required to see whether we are able to distinguish between the two models proposed for the sulfonium ion linkage. It is clear from our results that difference FTIR may become a powerful technique in specific detection of isotopically labeled single residues, in particular when combined with site-directed mutagenesis studies.

The second question is whether an enzyme is required in MPO for the formation of this special methionine sulfonium ion linkage or whether it is formed autocatalytically, as has been proposed for the two ester linkages of the heme group the Asp252 and Glu242 residue (52). The fact that active MPO can only be expressed by a mammalian cell line, such as CHO, might suggest that production of this enzyme requires some additional cofactors present in higher organisms.

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 (13) and distortion from the planar conformation of the heme 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 neighboring Glu242 residue (32). It is the combination of these two neighboring covalent linkages that induces the asymmetry in the heme macrocycle and may place the heme group in the right spatial configuration.

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