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Characterization of the Asp94 and Glu242 mutants in myeloperoxidase, the residues linking the heme group via ester bonds

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The heme group of all mammalian peroxidases is covalently linked to the protein matrix via two esterbonds, as we have recently shown by Fourier transform infrared (FTIR) difference spectroscopy [Kooter, I.M., Pierik, A.J., Merkx, M., Averill, B.A., Moguilevsky, N., Bollen, A. & Wever, R. (1997) *J. Am. Chem. Soc.* **119**, 11542–11543]. We have examined the effects of mutation of Asp94 and Glu242, responsible for those ester bonds in myeloperoxidase, on the spectroscopic properties and catalytic activity of this enzyme. Mutation of Asp94 in myeloperoxidase results in two species. The first species has spectroscopic characteristics similar to that of wild-type myeloperoxidase. The second species has spectroscopic characteristics similar to that of Met243→Gln mutant, and it is therefore concluded that, besides loss of the ester bond involving Asp94, this species also has lost the sulfonium ion linkage that is also characteristic of myeloperoxidase. The Asp94→Asn mutant still has about 30% residual peroxidase activity while for the Asp94→Val mutant only a few percentage activity is left. When Glu242 is mutated the sulfonium ion linkage is not affected, but this residue together with its neighbouring residue Met243, according to resonance Raman spectra, is responsible for the low symmetry of the heme group. Mutation of either of these residues results in loss of the bowed distortion from the planar conformation, and in a heme group with higher symmetry. For the Glu242→Gln mutant 8% residual peroxidase activity is found.

Keywords: myeloperoxidase; lactoperoxidase; unusual heme; ester bond.

As a member of the mammalian peroxidase family, myeloperoxidase (MPO) distinguishes itself in two ways from the other mammalian peroxidases, eosinophil peroxidase (EPO), lactoperoxidase (LPO) and thyroid peroxidase (TPO). First of all, it is the only member of this family that is able to peroxidize chloride to hypochlorous acid, a bactericidal agent, at a substantial rate [1]. Secondly, it differs in its spectroscopic characteristics. For example, the Soret band in the optical absorbance spectrum is red shifted to 428 nm, compared to 412 nm for the other mammalian peroxidases [2,3]. The resonance Raman spectrum is complicated [4,5] and monochlorodimedon (MCD) data show an inverted sign band pattern in the Soret region [6,7]. Those differences have been attributed to the heme group of MPO. However, a remarkable similarity is found between MPO and the other mammalian peroxidases. MPO shares, respectively, 70, 61 and 47% identical residues with EPO, LPO and TPO [8–10] and an even higher similarity can be found among the active site related residues [11]. MPO is the only mammalian peroxidase for which a crystal structure is known. Three residues are in close proximity to the heme group. Based on this, a novel derivative of iron protoporphyrin IX was suggested in which three covalent bonds are formed with the protein [12]. Two hydroxylated methyl groups on pyrrole rings A and C were claimed to form ester bonds with Glu242 and Asp94, while a sulfonium ion linkage was proposed between the sulphur atom of Met243 and the vinyl group on ring A [12,13]. This third linkage, involving the Met243 residue which is only found in MPO, was proposed to be responsible for the particular characteristics of MPO. We have recently shown that mutation of this Met243 into a glutamine results in a LPO-like protein [14].

Conservation of the Asp94 and Glu242 residues throughout the mammalian peroxidase family [11] had led to the suggestion that the ester bonds to the heme may be a common feature of members of this gene family [12,15]. Proteolytic digestions of LPO resulted in the indentification of an iron protoporhyrin IX structure whith hydroxylated methyl groups on positions 1 and 5 [16]. DePillis *et al*. [17] showed that the heme group in LPO is autocatalytically esterified to the aspartate and glutamate residues in the presence of H_2O_2. With help of Fourier transform infrared (FTIR) difference spectroscopy, we have recently provided the first direct evidence that the ester bonds are indeed present in all the mammalian peroxidases. Two sets of carboxylic ester C=O and C–O-group absorptions were observed, the positions of which are influenced by the redox state of the enzyme. Site-directed mutagenesis of MPO allowed them to be assigned specifically to the Glu242 and Asp94 residues [18]. In a recent study Rae and Goff showed that the heme-protein ester linkages in LPO occur between the two hydroxyl groups of the heme group,

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Abbreviations: MPO, myeloperoxidase; EPO, eosinophil peroxidase; LPO, lactoperoxidase; TPO, thyroid peroxidase; MCD, monochlorodimedon (1,1-dimethyl-4-chloro-3,5-cyclohexanedione); ABTS, 2,2′-azino-bis-(3-ethyl-benzo-6-thiazosulfonic acid); FTIR, Fourier transform infrared.

Enzymes: myeloperoxidase (EC 1.11.1.7).

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called heme *l*, and the carboxylic acid side chains of Glu275 and Asp125 [19].

In this study we further investigated the mutants wherein the Asp94 is replaced by an asparagine or a valine, and we reinvestigated the mutant in which Glu242 is replaced by a glutamine. We show that mutation of Asp94 results in two species, one with similar spectroscopical properties as that of native MPO and the other has characteristics of Met243Gln mutant, indicating that the sulfonium ion linkage is lost in part of the enzyme molecules. Surprisingly the Asp94→Asn still has about 30% residual peroxidase activity while for the Asp94→Val mutant only a few percentage activity is left. Mutation of Glu242 has a drastic effect on the spectral properties, and the symmetry reducing effects in the resonance Raman spectra were lifted. For this mutant, 8% residual peroxidase activity is still found.

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 myeloperoxidase were described in detail previously [20]. The Met243→Gln, Asp94→Asn, Asp94→Val and Glu242→Gln mutant proteins were produced by replacing, in the myeloperoxidase-coding cassette carried by plasmid pNIV2703, a 178-bp *Apa*I/*Avr*II DNA fragment by the mutated counterpart. The final plasmids were called pNIV2718, 2713, 2729 and 2714, 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 (US Biochemical Corp.). The final recombinant plasmid was transfected into Chinese hamster ovary cells, and G418-resistant colonies were selected and expanded. Cell factories supernatant (10 L) was collected, and the mutant was purified [20].

It was found that the recombinant MPO had a lower R_z value ($A_{428\ nm}/A_{280\ nm}$) than native MPO and also the mutants 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 native MPO of 89 $mM^{-1}\cdot cm^{-1}$.

All optical spectra for the mutants were recorded on a Cary 50 Biospec spectrophotometer. Dithionite solution was used for reduction of the oxidized species. The pyridine hemochrome spectra were prepared in 2.1 M pyridine and 75 mM NaOH, and a concentrated dithionite solution was added for reduction.

EPR measurements at the 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 earlier [14].

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 $mM^{-1}\cdot cm^{-1}$ [21]) into dichlorodimedone (ε = 0.2 $mM^{-1}\cdot cm^{-1}$ at 290 nm). The chlorinating activity was also measured by monitoring the formation of taurine monochloramine at 252 nm (ε = 429 $M^{-1}\cdot cm^{-1}$ [22]). The guaiacol assay was performed by monitoring the formation of tetraguaiacol at 470 nm (ε = 26.6 $mM^{-1}\cdot cm^{-1}$ [23]). The 2,2′-azino-bis-(3-ethyl-benzo-6-thiazosulfonic acid) (ABTS) assay was performed by monitoring the formation of the oxidation product at 414 nm (ε = 36.0 $mM^{-1}\cdot cm^{-1}$ [24]). The assays were performed with 2.5 mM ABTS, 8 mM guaiacol, 50 μM MCD or 15 mM taurine. All assays were performed in 100 mM potassium phosphate (pH 7) or sodium acetate (pH 5) and 200 mM Na_2SO_4, and with 5–50 nM MPO and 100 μM H_2O_2. 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 H_2O_2. We decided not to explore the kinetic parameter (V_{max} and K_m) values in detail considering the complex kinetic behaviour of MPO [25].

RESULTS

Expression of the cDNA of human MPO in Chinese hamster ovary cell lines [20] results in the secretion of a 84-kDa monomeric single-chain precursor with spectroscopic and

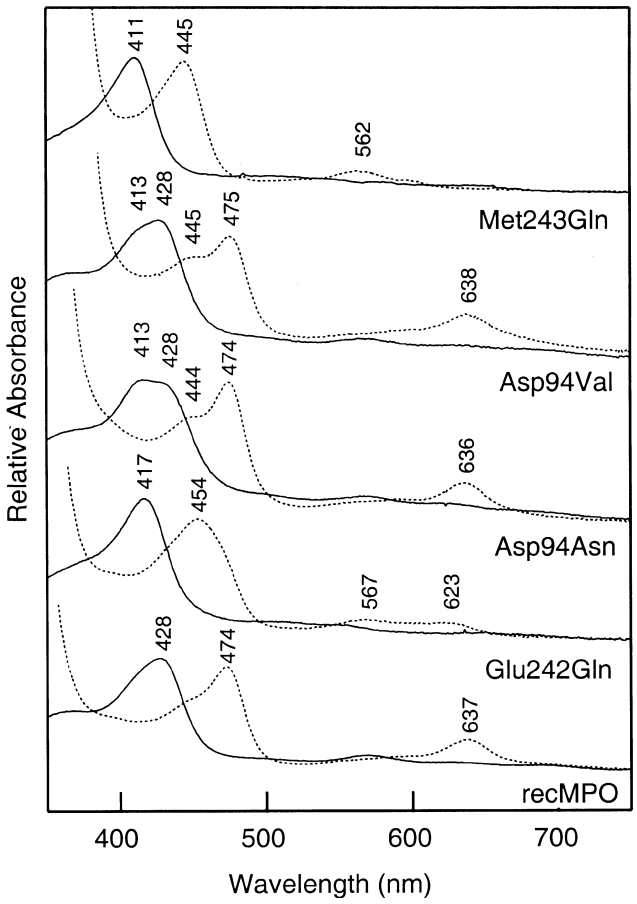

Fig. 1. Optical absorbance spectra of the Met243→Gln, Asp94→Val, Asp94→Asn, and Glu242→Gln mutant, and recombinant MPO. Met243→Gln mutant in 100 mM pyrophoshate buffer (pH 9), Asp94→Val, Asp94→Asn, and Glu242→Gln mutant, and recombinant MPO in 100 mM potassium phosphate buffer (pH 7). Oxidized, solid line; reduced, dotted line.

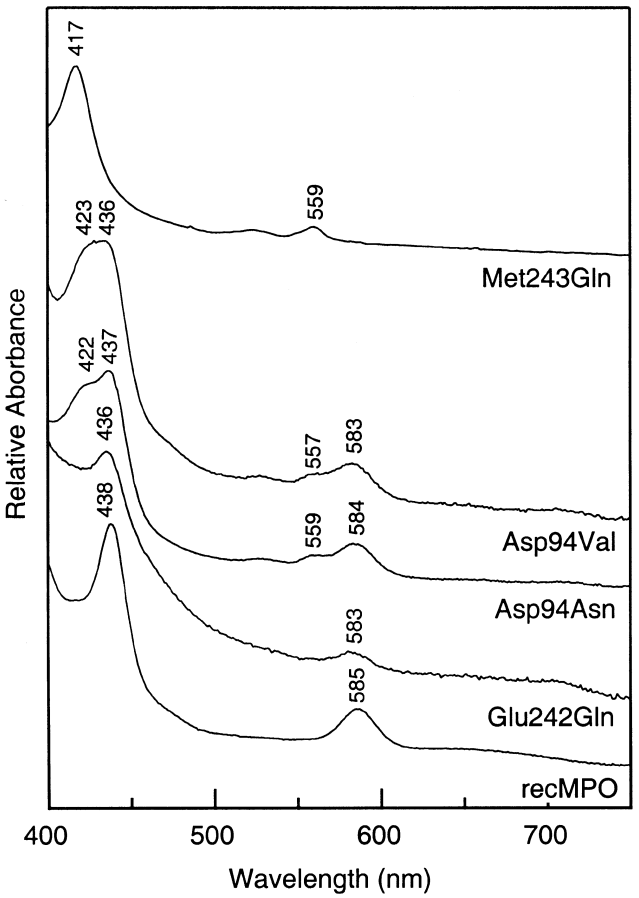

Fig. 2. Alkaline pyridine hemochrome spectra of the Met243→Gln, Asp94→Val, Asp94→Asn, and Glu242→Gln mutant, and recombinant MPO. The alkaline pyridine hemochrome spectra were prepared in 2.1 M pyridine and 75 mM NaOH, and a concentrated dithionite solution was added for reduction.

kinetic properties similar to that of the native dimeric two-chain enzyme [14,26,27]. Site-directed mutagenesis enables us to study mutants of the ester bond forming residues Asp94 and Glu242 by spectroscopic and kinetic techniques. In an earlier study of the Glu242→Gln mutant [27], it was concluded that the negatively charged carboxylate group of Glu242 interacted with the prosthetic group of MPO. This interaction would then partly be responsible for the red shifted Soret band in the optical absorbance spectrum and be the origin of the symmetry-reducing effects in the resonance Raman spectrum of the native enzyme. As the Glu242 was shown to be involved in a heme-linking ester bond [18], and mutation of the Met243 into a glutamine resulted in similar resonance Raman spectra as that of the Glu242→Gln mutant [14] but in different pyridine hemochrome spectra, we decided to reinvestigate the Glu242→Gln mutant.

Figure 1 shows the optical spectrum of the Met243→Gln, Asp94→Val, Asp94→Asn, and Glu242→Gln mutant, and recombinant MPO. The spectrum of the recombinant MPO is similar to that of the native enzyme in its oxidized and reduced state (not shown), as has been shown before [26]. The recombinant MPO in which Asp94 is replaced by a valine (Asp94→Val mutant) shows a rather broad Soret band in the oxidized state around 424 nm, in which two maxima can be observed at 413 and 428 nm. Reduction of this mutant results in a more clearly resolved spectrum, with maxima at 445 and 475 nm and an α band at 638 nm. For the Asp94→Asn mutant, very similar spectra are found for both the oxidized state and reduced state. One set of Soret bands (oxidized 428 nm, reduced 474 nm) is similar to native MPO, whereas the other set of bands (oxidized 413 nm, reduced 445 nm) is similar to the spectrum of the Met243→Gln mutant MPO, Fig. 1 and [14]. Reduction of Asp94→Asn by sodium dithionite in the presence of 0.5 μM methyl viologen for a period of 1 h did not change the spectrum, indicating that the sample was completely reduced. Mutation of the second ester bond-forming residue, Glu242, into a glutamine results in an optical spectrum that has one symmetrical Soret band at 417 nm for the oxidized enzyme state. For the reduced state one symmetrical Soret band at 454 nm and minor bands at 567 and 623 nm are found. These spectra are similar to those reported before for this mutant [27].

Pyridine hemochrome spectra are often used to give an indication of the type of heme present in the protein. The high pH disrupts the noncovalent interactions between the protein and the heme group, and pyridine replaces the axial ligands of the heme group, forcing the heme into a single ligation state. Figure 2 shows that the pyridine hemochrome spectrum of the Asp94→Val mutant has two Soret bands at 423 and 436 nm, and two α bands at 557 and 583 nm. The Asp94→Asn mutant shows a more clearly resolved spectrum with two Soret bands at

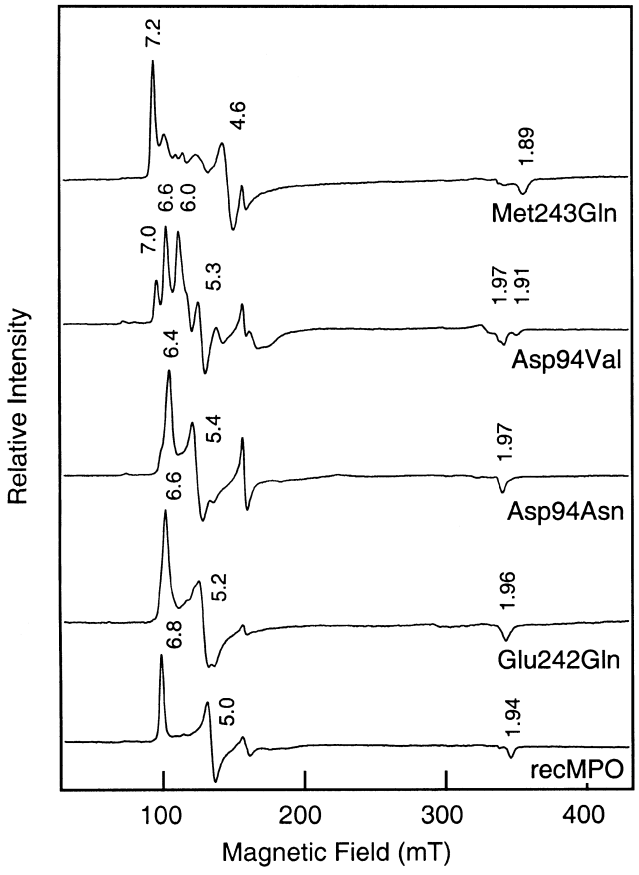

Fig. 3. EPR spectra of the high-spin forms of Met243→Gln, Asp94→Val, Asp94→Asn, and Glu242→Gln mutant, and recombinant MPO. Met243→Gln (80 μM), Asp94→Val (65 μM), Asp94→Asn (150 μM), Glu242→Gln (45 μM) and recombinant MPO (29 μM). All samples in 100 mM potassium phosphate buffer (pH 7). Conditions during 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.

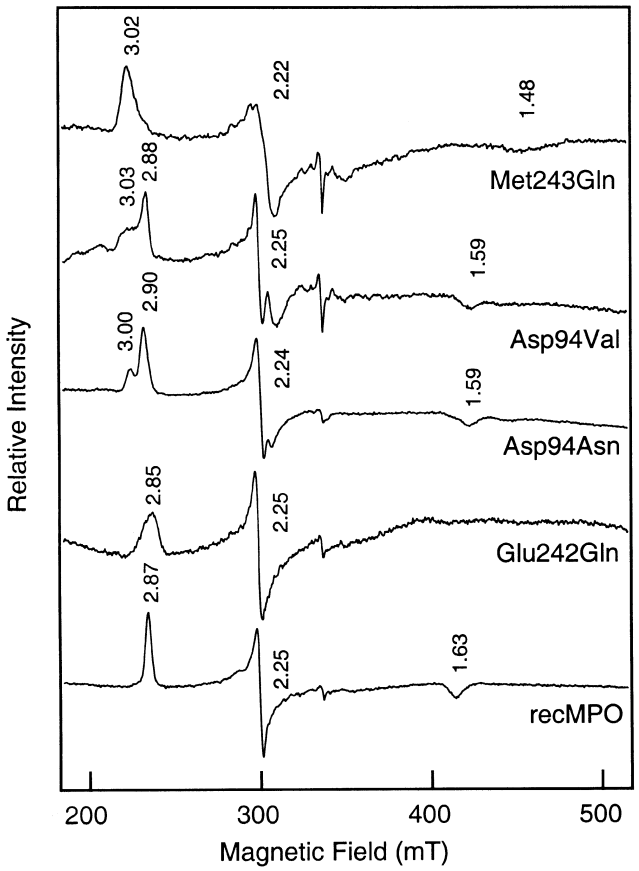

Fig. 4. EPR spectra of the low-spin forms of Met243→Gln, Asp94→Val, Asp94→Asn, and Glu242→Gln mutant, and recombinant MPO. Low-spin states were obtained by addition of potassium cyanide solution (0.5 M final concentration) in 100 mM sodium carbonate buffer (pH 9.5) to the Met243→Gln mutant MPO of Fig. 3, and by addition of potassium cyanide solution (10 mM final concentration) in 100 mM sodium carbonate buffer (pH 9.5) to the Asp94→Val, Asp94→Asn, and Glu242→Gln mutant, and recombinant MPO of Fig. 3. Conditions during the recording of the spectra were as in Fig. 3.

422 and 437 nm, and two α bands at 559 and 584 nm. The presence of two Soret bands in the spectra of the Asp94 mutants is indicative of the presence of two different species. One species corresponds to recombinant MPO whereas the other is similar to that of Met243→Gln (Fig. 2). The Glu242→Gln mutant shows a single Soret band at 436 nm, and a α band at 583 nm, which, as reported before, are similar in position as found for the pyridine hemochrome of recombinant MPO system [27].

Figure 3 shows the typical high-spin heme EPR spectra of the Met243→Gln, Asp94→Val, Asp94→Asn and Glu242→Gln mutant, and recombinant MPO. The Met243→Gln mutant shows a major component with g_x,g_y,g_z = 7.2, 4.6, 1.89 and at least one minor species. The Asp94→Val mutant shows several high-spin signals (g_x = 7.0, 6.6, 6.0), indicating a mixture of species. The Asp94→Asn mutant also shows an additional weak high-spin signal with g_x = 7.0. The spectrum of the Glu242→Gln mutant also contains an additional signal, most clearly visible in its split g_y signal. This signal is also present in the spectrum of the Glu242→Gln mutant reported previously [27]. High-spin spectra are known to be sensitive to small conformational differences [28]. For this reason we investigated the low-spin state of the mutants induced by addition of 10 mM

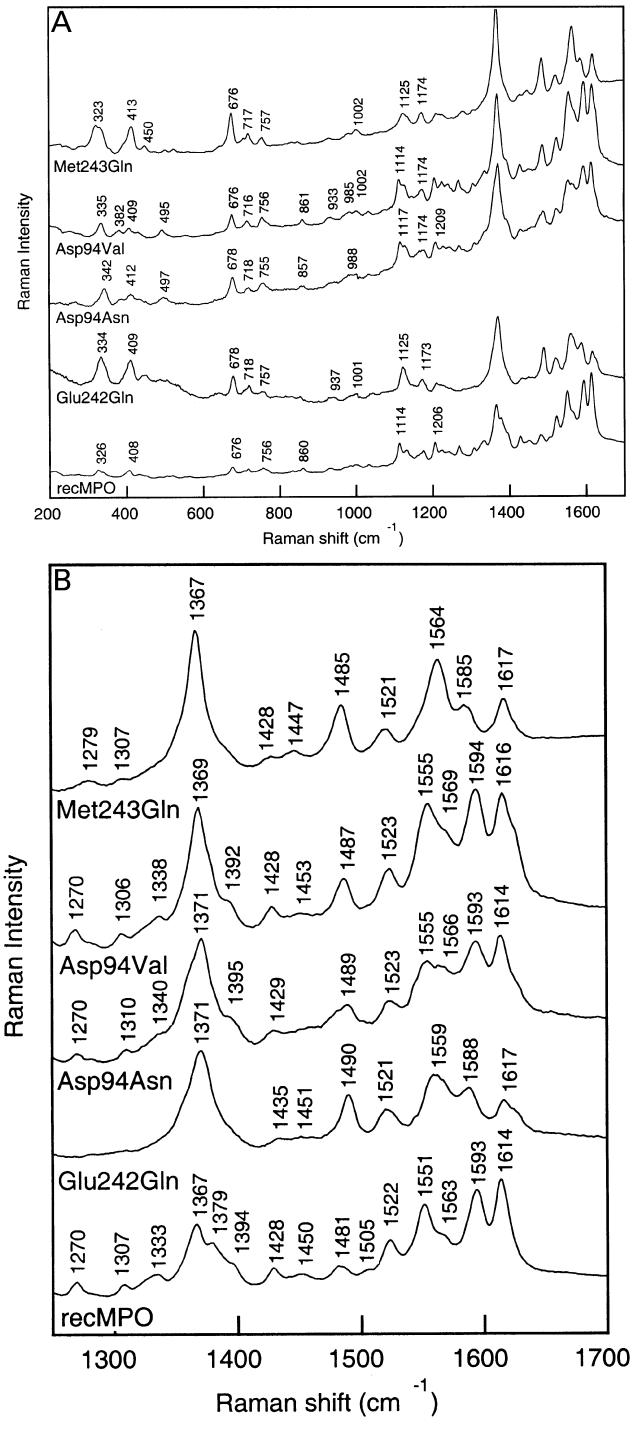

Fig. 5. Resonance Raman spectra of Met243→Gln, Asp94→Val, Asp94→Asn, and Glu242→Gln mutant MPO, and recombinant MPO in the 200–1700 cm^{-1} (A) and 1250–1700 cm^{-1} (B) frequency regions. Met243→Gln (80 μM) (acquisition time 3 × 100 s, laser power in sample 5 mW), Asp94→Val (60 μM) (acquisition time 2 × 200 s, laser power in sample 5 mW), Asp94→Asn (150 μM) (acquisition time 10 × 300 s, laser power in sample 7 mW), Glu242→Gln (45 μM) (acquisition time 6 × 300 s, laser power in sample 7 mW), recMPO (30 μM) (acquisition time 2 × 200 s, laser power in sample 5 mW). Raman spectra were obtained with 413.1 nm excitation wavelength. Samples were in 100 mM potassium phosphate (pH 7.0).

Table 1. Activity of MPO and mutants. Conditions are as described under Materials and methods. Activity is expressed in s^{-1} and was calculated from the absorbance changes and corresponding extinction coefficients. ND, not determined.

	Guaiacol		ABTS		MCD		Taurine	
Enzyme	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	6.5	129	0.7	7	4.6	6.6
Met243→Gln	0.9	0.17	0.28	1.2	ND	0.4	< 0.1	< 0.1
Asp94→Val	6.8	0.44	3.8	5.5	ND	0.1	< 0.1	< 0.1
Asp94→Asn	46	7.3	14.5	32.1	0.4	4.5	0.2	2.2
Glu242→Gln	7.8	4.4	9.4	14.3	0.1	1.7	0.3	0.5

cyanide. Figure 4 shows the low-spin state of the Met243→Gln, Asp94→Val, Asp94→Asn and Glu242→Gln mutant, recombinant MPO. It is clear that in both Asp94 mutants, two low-spin species are present, one with g_x = 3.0 and the other with a less rhombic signal with g_x = 2.9. The first signal resembles that of the cyanide complex of recombinant MPO, whereas the latter resembles that of the cyanide complex of a recombinant MPO in which the Met243 residue has been mutated. The low-spin EPR spectrum of the Glu242→Gln mutant MPO shows signals similar in position to that of the recombinant MPO, but which are considerably broader, indicating more *g*-strain.

The resonance Raman spectrum of MPO is complex and shows multiple bands in the oxidation state marker region (ν_4 = 1367 cm^{-1}). These characteristics have been attributed previously to the symmetry-lowering effects of a chlorin-like structure [4,5,29]. The Met243→Gln has a resonance Raman spectrum similar to that of the mammalian peroxidases LPO and EPO and indicative of an iron protoporphyrin IX as the prosthetic group [14]. Similar results have been found for the Glu242→Gln mutant [27]. Figure 5A,B shows the resonance Raman spectra of the Met243→Gln, Asp94→Val, Asp94→Asn and Glu242→Gln mutant, and recombinant MPO. The spectra of the Asp94→Val and the Asp94→Asn mutant are similar in shape and intensity. It is apparent that the resonance Raman spectra of the Asp94 mutants show features of both the recombinant MPO and the Met243→Gln mutant. Especially in the higher frequency region (Fig. 5B), bands similar to both the recombinant and Met243 mutant are present in the Asp94 mutant MPO, and also the ν_4 ($\approx$1370 cm^{-1}) band has some asymmetric character. The Glu242→Gln mutant MPO has a less complex spectrum, with a highly symmetric ν_4 line at 1371 cm^{-1}. The bands in the high frequency region, between 1450 and 1650 cm^{-1}, are affected both in their intensity and positions.

Within the mammalian peroxidases family, MPO is the only peroxidase that has the ability to peroxidize chloride to hypochlorous acid at a substantial rate. The recombinant MPO has the same kinetic parameters in the chlorination reaction as found for the native MPO [26,27]. The chlorination activity of MPO is often determined by following the reaction between HOCl and MCD, which results in a decrease of extinction at 290 nm due to formation of the dichlorocompound. In some of the mutants, a decrease of the extinction at 290 nm was observed even in the absence of chloride, similar to that observed with native MPO in the absence of a halide substrate [1,30]. This indicates that some mutants were capable of direct oxidation of MCD in the presence of hydrogen peroxide. For this reason we also used the chlorination of taurine as an alternative chlorination activity assay for the mutants as it is known that taurine is unreactive toward the MPO compounds I and II [31,32]. Two classical peroxidase substrates were also investigated. Table 1 shows the activity for the different assays as measured at pH 7 and 5. For native MPO, the pH optimum in the guaiacol assay is at pH 7, whereas that for the ABTS assay was around pH 5. As mutation of Met243 results in 1% residual classical peroxidase activity [14], it is assumed that the Met243 mutant-like part of the Asp94 mutants does not contribute to the activity. The activities for the Asp94 mutants are therefore calculated using the concentration based on the extinction at 428 nm. This wild-type-like fraction is considered to be completely responsible for the observed activity. The Asp94→Val mutant and Asp94→Asn mutant differ in their enzyme activity. For the classical peroxidase substrates, a residual activity of about 5% is found for the Asp94→Val mutant, while approximately 30% residual activity is found for the Asp94→Asn mutant (at the optimum pH conditions of native MPO). The chlorination activity using the taurine assay at pH 5 showed that the Asp94→Asn mutant had 33% activity of the wild-type, and a less than 2% residual activity is found for the Asp94→Val mutant. The activity measured by the MCD assay at this pH results in higher values, probably due to direct oxidation of MCD by the mutants. The Glu242→Gln mutant shows approximately 8% classical peroxidase activity and 7% chlorination activity as found by the taurine assay. Previously it was reported that this mutant had no chlorinating activity [27]. It is apparent that for the native and recombinant MPO system the turnover numbers of both chlorination assays at pH 5 are similar, whereas differences appear at pH 7.

DISCUSSION

In the family of mammalian peroxidases, MPO has an eccentric position due to its unique ability to peroxidize chloride and its unusual spectral properties. These characteristics have been attributed to the heme group. The Asp94 and Glu242 residues, conserved throughout the mammalian peroxidase family, together with the nonconserved Met243 residue, have been proposed to form covalent linkages with the heme group. Recently we have presented the first direct evidence that Asp94 and Glu242 form ester bonds to the heme in all mammalian peroxidases [18]. In light of these findings we now have further characterized the Asp94 mutants. We also reinvestigated the Glu242→Gln mutant because the suggestion that the Glu242 residue influences the chromophore via its negatively charged carboxylate group [27] was shown to be incorrect [18].

Mutation of the Asp94 of MPO into either a valine (Asp94→Val mutant) or an asparagine (Asp94→Asn mutant)

results in a protein with two Soret bands, both in the oxidized and in the reduced form of the optical absorbance spectrum and in the pyridine hemochrome spectrum. One band seems to correspond to that of recombinant MPO, whereas the position of the other is similar to that of Met243→Gln mutant ([14], and this study). Although the EPR spectra indicate the presence of several high-spin species, it is clear that in the low-spin cyanide complex of the Asp94 mutants, two species are present. The resonance Raman spectrum of both the Asp94 mutants seems to be a mixture of wild-type-like species and a Met243→Gln mutant-like species. Thus, optical absorbance, EPR and resonance Raman spectroscopy all demonstrate that mutation of the Asp94 results in two species. One species has the characteristics of native MPO while the second possesses those of a Met243 mutant species, in which the sulfonium ion linkage can not be formed. Thus in addition to the loss of the Asp94 ester bond in the second species, the Met243 sulfonium ion linkage seems not to be present. Either this linkage is not formed during the processing of this mutant, or this linkage is not very stable in the absence of the Asp94 ester bond. Because the Met243 sulfonium ion linkage seems to be at least partially present, we prefer the latter explanation.

Thus, the Asp94 mutants consist both of an Asp94 mutant species in which only the Asp94 is mutated and a second Asp94 mutant species in which also the Met243 sulfonium ion linkage is lost. Apparently, loss of the Asp94 ester bond destabilizes the sulfonium ion linkage.

The loss of the Asp94 ester bond only, has no effect in the optical absorbance of the oxidized and reduced states of this mutant. In contrast, the loss of the Glu242 ester bond results in a blue-shift in the optical spectra of both the oxidized and reduced enzyme states. Apparently the two ester bonds are not equivalent in their effect on the spectral properties of the heme. This may indicate that it is not the loss of the ester bond itself that causes this shift in the optical spectrum of the Glu242→Gln mutant. The pyridine hemochrome spectra of both the first Asp94 mutant species and Glu242 mutants are similar to that of the recombinant enzyme state. This might be a result of the alkaline conditions used in this experiment hydrolysing both ester bonds, or more likely, the presence of ester bonds not affecting the pyridine hemochrome spectrum because they are nonconjugated substituents. The fact that both the Asp94 and the Glu242 mutations result in the same pyridine hemochrome spectra indicates that the mutants have a heme group with the same chemical nature as the native enzyme, despite the differences suggested by their optical spectra (oxidized and reduced form). In a previous study, the blue-shift in the Glu242→Gln mutant was explained by the loss of the negatively charged carboxylate group of Glu242, which could interact with the prosthetic group of MPO. This interaction would partly be responsible for the red-shifted Soret band in the optical absorbance spectrum [27]. Because we now know that Asp94 and Glu242 make ester bonds to the heme group, this assumption is shown to be incorrect. The blue-shift of the Soret band from 474 to 454 nm in the reduced state of the Glu242→Gln mutant MPO in the optical absorbance spectrum may therefore be a result of a rearrangement of the bowed heme structure [12], resulting in a more flexible heme of higher symmetry. The red-shifted position of the Soret band of the Glu242→Gln mutant (e.g. 454 nm in the reduced state) as compared to that of LPO (e.g. 446 nm in the reduced state) is then the result of the electron-withdrawing effect of the sulfonium ion linkage. This linkage is still present in this mutant as shown by the fact that it is still sensitive to autocleavage of the Met243-Pro244 bond [14]. Future mutation studies of the ester forming residues, Asp125 and Glu275, in LPO are important to further investigate the origin of the red-shift in the Soret band of optical absorbance spectra of mammalian peroxidases.

The EPR spectra of the low-spin Glu242→Gln and Met243→Gln mutants are indicative of *g*-strain, as can be seen by the broader g_x signal. This indicates more flexibility of the heme iron and microheterogeneity of the protein conformation [33,34]. Resonance Raman spectra of native MPO have been interpreted as indicative of a heme group with a low symmetry, such as an iron chlorin [4,5,29]. The spectrum is rather complex, especially in the oxidation-state marker region (ν_4), where multiple lines arise due to symmetry reduction. It is now clear that these symmetry-lowering characteristics seen in MPO by resonance Raman are due to the two neighbouring residues. Both residues, Glu242 and Met243, appear to put the heme group in a fixed conformation with a low symmetry. A more symmetric heme group is obtained by either breaking the Glu242 ester bond or the Met243 sulfonium ion linkage, resulting in both cases, in a more symmetric familiar iron protoporhyrin IX-like resonance Raman spectrum.

Chlorination activity is completely lost when Met243 is mutated ([14], and this work). Mutation of the Glu242 results in a dramatic loss of chlorination, although the mutant is not completely inactive as has been reported previously [27]. Thus, the presence of the sulfonium ion linkage seems to be essential for chlorinating activity and the neighbouring heme-linking residue Glu242, seems to further optimize the chlorination activity. We conclude that both heme-linking residues Glu242 and Met243 put the heme group in a solid fixed conformation with a low symmetry. Mutation of either of these residues results in loss of this conformation.

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