Heme−Protein Interaction in Myeloperoxidase: Modification of Spectroscopic Properties and Catalytic Activity by Single Residue Mutation†

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Abstract: The optical absorbance spectrum of reduced myeloperoxidase shows an unusual red-shifted Soret band at 472 nm and an α band at 636 nm. It has been speculated that this red-shift is due to interaction of the protein matrix with the chromophore. The carboxylate side chain of Glu242 is in close proximity of the prosthetic group of the enzyme, and we have examined the effect of the Glu242 to Gln mutation on the spectroscopic properties and catalytic activity of the enzyme. The mutation shifts the Soret band in the optical absorption spectrum of the reduced mutated enzyme from 472 to 458 nm. The EPR spectrum was hardly affected and was typical of a rhombic high-spin system ($g_x = 6.6$, $g_y = 5.2$). The alkaline pyridine hemochrome spectrum of the mutant was nearly identical to that of native myeloperoxidase. The resonance Raman spectrum, however, was drastically affected in the mutant. The symmetry-reducing effects were lifted by the mutation and the resonance Raman spectrum was indicative of an iron-porphyrin-like chromophore with a singlet ν_4 line at 1367 cm^{-1}. The mutant enzyme was not able to peroxidize chloride to hypochlorous acid. We conclude that the interaction of residue Glu242 with the prosthetic group in native myeloperoxidase is partly responsible for the red-shifted Soret band in the optical spectrum and that this interaction is the origin of the symmetry-reducing effects in the resonance Raman spectrum of the native enzyme. This residue also plays a pivotal role in the ability of the enzyme to peroxidize chloride.

Introduction

Myeloperoxidase (EC 1.11.1.7) is present in high concentration in azurophilic granules of polymorphonuclear neutrophils.[1,2] The enzyme has an important role in the antimicrobial activity of the neutrophils, since the enzyme catalyzes the peroxidation of chloride by hydrogen peroxide to form hypochlorous acid,[3−5] which is a bactericidal agent.[6−8] The prosthetic group of myeloperoxidase is a heme group, the chemical nature of which is not identified, due to the fact that it is not possible to extract the heme which is covalently linked to the protein.[9−11] The optical absorbance spectra of myeloperoxidase are rather peculiar,[12,13] e.g. the reduced enzyme displays a Soret band at 472 nm and the α band at 636 nm, which are red-shifted as compared to those of other hemoproteins. Pyridine hemochrome spectra of myeloperoxidase[9,12,14] and the spectra of the enzyme treated with sodium dodecyl sulfate and acid[13,15] are similar to spectra of heme a containing cytochrome *c* oxidase. In addition, carbonyl reagents were shown to react with the enzyme,[9,12] which indicates the presence of a formyl group, like in heme a. Therefore, it has been suggested that the prosthetic group of myeloperoxidase is similar to heme a. On the other hand, a formyl group has not been detected by resonance Raman spectroscopy.[16] There are other studies that indicate that the prosthetic group is a porphyrin.[17−20] However, opposed to these suggestions, both magnetic circular dichroism[21,22] and resonance

† This paper is dedicated to the memory of Professor Bob van Gelder who has contributed so much to studies of oxido-reductases in The Netherlands.

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Raman studies[23-26] have suggested an iron chlorin as the prosthetic group of myeloperoxidase.

It has not yet been possible to explain all the spectroscopic properties of the enzyme, and it was suggested that the protein part interacts with the chromophore to affect the electronic structure of the heme.[27] Recent denaturation/renaturation studies[27,28] suggested that a protonatable negatively charged residue is present close to the chromophore which interacts with the heme group to impose the red-shift in the optical spectrum. A major problem with the denaturation studies is that the properties of the enzyme are affected in a nonselective manner. However, that specific side chains of amino acids affect the spectral properties of macrocycles has also been proposed recently with regard to the optical spectra of bacteriochlorophylls,[29,30] which are present in light-harvesting complexes of purple bacteria.

The X-ray crystal structure of canine myeloperoxidase has been determined to 3 Å resolution,[31] but it was not possible to determine the exact structure of the heme group with these data. However, the X-ray analysis indicates that two carboxylate groups (Glu242 and Asp94) are indeed present close to the chromophore, and from the low-resolution X-ray data of myeloperoxidase, it was suggested that Glu242 shares electron density with the prosthetic group. Recently, it was shown that it is possible to express the cDNA of human myeloperoxidase in Chinese hamster ovary (CHO) cell lines[32] and that a 84 kDa monomeric single-chain precursor is secreted with physiochemical properties which are in many respects identical to those of the mature enzyme.[33] It has also been possible to carry out single residue mutations in this enzyme, and some evidence that the residues Glu242 and Asp94 interact closely with the periphery of the heme has been obtained by using site-directed mutagenesis of the human enzyme.[34] Here we report a detailed study in which we have replaced Glu242 by Gln and thus specifically removed the negative charge. This mutation has a large effect on the optical, resonance Raman and catalytic properties of the enzyme. We conclude that a charged group in the vicinity of the heme macrocycle of myeloperoxidase greatly affects its electronic properties.

Experimental Section

Human myeloperoxidase was isolated and purified from leukocytes as described previously.[35] The enzyme concentration was determined from the optical absorbance at 428 nm using an absorbance coefficient of 89 $mM^{-1}\cdot cm^{-1}$. Bovine lactoperoxidase was purchased from Sigma Chemical Co. Peripheral blood eosinophils were isolated according to the method described previously.[36]

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.[32] The Glu242 to Gln mutant protein was produced by replacing, in the myeloperoxidase-coding cassette carried by plasmid pNIV2703,[32] a 178 bp ApaI-AvrII DNA fragment by the mutated counterpart. The final plasmid was called pNIV2714.[34] The mutation was generated within this fragment by a combination of polymerase chain reactions[37] and overlap extensions, using an oligonucleotide primer carrying the modified codon. The amplified fragment was sequenced using Sequenase version 2 (U.S. Biochemical Corp.). The final recombinant plasmid was transfected into Chinese hamster ovary cells, and G 418 resistant colonies were selected and expanded in 175 cm^2 Falcon flasks. Cell culture supernatant was collected, and the mutant was purified.[32,38]

The optical spectra of the oxidized and reduced species were recorded on a Hewlett Packard 8452 A diode array spectrophotometer, and dithionite was used for reduction. The EPR spectra were recorded on a Varian E-9 spectrometer. The resonance Raman spectra were recorded using a confocal Raman microspectrometer[39-41] which was adapted for the experiment using 413.1 nm excitation. The most important adaptation was the use of a liquid nitrogen cooled charge-coupled device (CCD) camera, fitted with a Tektronix 512 TKB thinned back-illuminated CCD chip, which shows a very good response in the blue spectral region (quantum efficiency at 400 nm of 60%, Princeton Instruments Inc., Trenton, NJ). Laser line suppression was achieved with a holographic notch filter (Kaiser Optical Systems, Inc., Ann Arbor, MI).

The Raman measurements were carried out with protein samples contained in square capillary glass tubes (inner diameter 500 μm, wall thickness 100 μm, Vitro Dynamics Inc., Rockaway NJ) using a 63× Zeiss Plan Neoflar water immersion objective with cover glass correction. A laser power of 0.4 mW was used.

The chlorinating activity was measured by monitoring the conversion of monochlorodimedone (1,1-dimethyl-4-chloro-3,5-cyclohexanedione, ϵ = 20.2 $mM^{-1}\cdot cm^{-1}$ at 290 nm) into dichlorodimedone (ϵ = 0.2 $mM^{-1}\cdot cm^{-1}$ at 290 nm).[42] The pyridine hemochrome spectra were prepared in 2.1 M pyridine and 75 mM NaOH, and a few grains of dithionite were added for reduction.

Results

The optical spectrum of oxidized human myeloperoxidase displays a Soret band at 428 nm and a band at 570 nm. The spectrum of the reduced enzyme is rather unique with a Soret band at 472 nm and an α band at 636 nm (not shown). The optical spectra of both the oxidized and the reduced forms of the monomeric single-chain recombinant myeloperoxidase are identical to those of native myeloperoxidase.[33] Thus, site-directed mutagenesis of recombinant myeloperoxidase can be used to study the effect of mutations on the spectral properties of myeloperoxidase.

Figure 1 shows the optical spectra of the recombinant myeloperoxidase variant in which the negatively charged residue Glu242 was replaced by Gln (E242Q variant). In the absorbance spectrum of the oxidized mutated enzyme, the Soret band is observed at 418 nm and weak bands are present at 512 and 580 nm. The spectrum of the reduced mutant displays a

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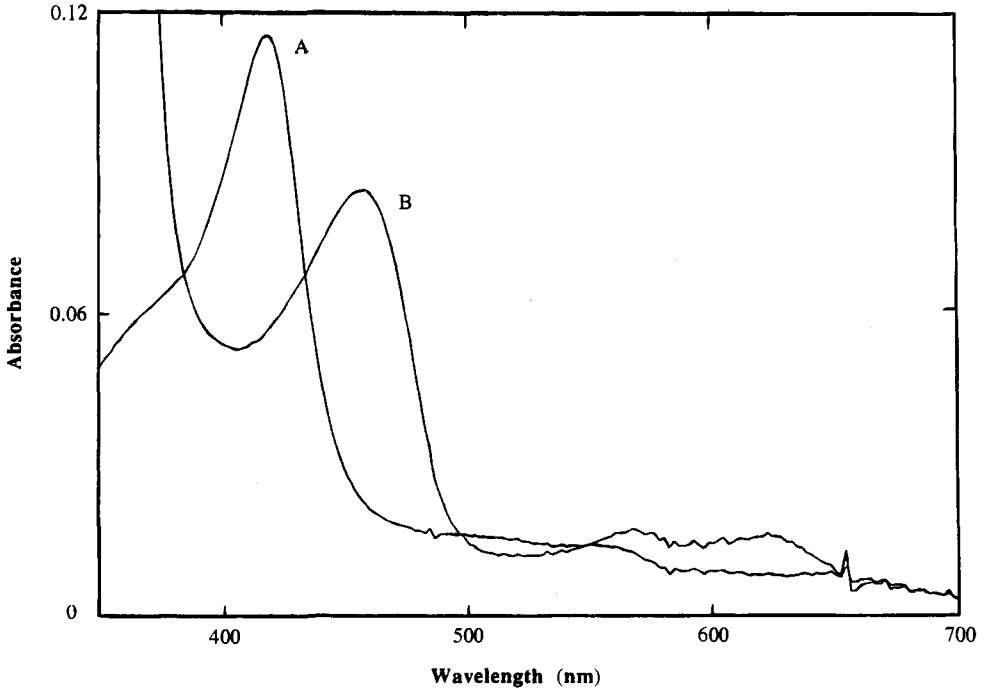

Figure 1. Optical absorbance spectra of the E242Q variant of myeloperoxidase: (A) oxidized enzyme in 100 mM sodium phosphate buffer (pH 7.0) and (B) reduced enzyme. The concentration of enzyme was estimated to be 1 μM.

symmetrical Soret band at 458 nm, and bands are present at 568 and 622 nm. The position of both the Soret band and the α band in the absorbance spectrum of reduced E242Q are blue-shifted by 14 nm as compared to that of the Soret band in the spectrum of native reduced enzyme.

Figure 2 shows the EPR spectra of native myeloperoxidase, recombinant myeloperoxidase, and the E242Q myeloperoxidase variant. The EPR spectra of both native ($g_x = 6.6$, $g_y = 5.2$) and recombinant myeloperoxidase ($g_x = 6.8$, $g_y = 5.0$) show typical high-spin rhombic signals, and no low-spin signals were detected. The rhombicity of the recombinant myeloperoxidase EPR signal is slightly higher than that of native myeloperoxidase. This may be due to the presence of chloride, which is known to affect the rhombicity of the EPR signal of myeloperoxidase.[43,44] The EPR spectrum of the mutant ($g_x = 6.6$, $g_y = 5.2$) is very similar to that of the native enzyme, and this shows that the mutation has hardly any effect on the rhombicity of the EPR signal. The $g = 4.3$ signals observed in the EPR spectra are due to adventitious iron.

Pyridine hemochrome spectra are widely used as an indication of the type of heme present in hemoproteins. The treatment at high pH will disrupt noncovalent interactions of the chromophore with the protein, and the axial ligands will be displaced by pyridine. Figure 3 shows the pyridine hemochrome spectra of cytochrome *c* oxidase, native myeloperoxidase, recombinant myeloperoxidase, and the E242Q variant. The pyridine hemochrome spectrum of native myeloperoxidase is very similar to that of cytochrome *c* oxidase with bands that are red-shifted as compared to iron protoporphyrin IX, which is in agreement with previous reports.[9,12,14] The pyridine hemochrome spectra of recombinant myeloperoxidase and the E242Q variant are almost identical to that of native myeloperoxidase. This experiment demonstrates that the mutation has hardly any effect on the optical properties of the pyridine hemochrome and that the interaction between the chromophore and the Glu242 is of a noncovalent nature.

Resonance Raman studies suggested a chlorin-type structure as the prosthetic group of myeloperoxidase.[16,23–26] These studies indicate that, due to symmetry-lowering effects of the chlorin-like structure, the resonance Raman spectrum of myeloperoxidase becomes more complicated with multiple bands in the ν_4 (oxidation-state marker) region. Figure 4 displays the resonance Raman spectra of the E242Q variant, recombinant myeloperoxidase, and native myeloperoxidase and for comparison also those of the mammalian peroxidases lactoperoxidase and eosinophil peroxidase at high spectral resolution. The resonance Raman spectrum of single-chain monomeric recombinant myeloperoxidase and of the dimeric native myeloperoxidase are almost identical and are more complex than those of lactoperoxidase and eosinophil peroxidase. The multiple bands around 1365 cm^{-1} (oxidation-state marker region), which are a characteristic feature in the resonance Raman spectrum of myeloperoxidase, are also observed in the recombinant myeloperoxidase spectrum. This shows that the structure of the prosthetic group of recombinant myeloperoxidase is identical to that of native myeloperoxidase and that the polypeptide is folded around the prosthetic group in a proper way.

Wever *et al.*[27] assumed that charged residues were also responsible for the asymmetry in the resonance Raman spectrum. Therefore, the effect of the Glu242 to Gln mutation on the resonance Raman spectrum was studied. The effect of the mutation on the resonance Raman spectrum is remarkable and is most obvious in the oxidation-state marker region (Figure 4, top panel). From the high-resolution high-frequency part of the resonance Raman spectra, it is clear that the mutation results in a highly symmetric ν_4 line at 1367 cm^{-1} similar in shape and position to those of lactoperoxidase and eosinophil peroxidase. It is evident that the resonance Raman spectrum of the E242Q variant is less complicated than those of recombinant myeloperoxidase and native myeloperoxidase. The only difference in the spectra is the relatively high intensity of the band at 1586 cm^{-1} in the spectrum of E242Q as compared to those of the bands in the spectra of lactoperoxidase (1589 cm^{-1}) and eosinophil peroxidase (1585 cm^{-1}). Figure 4 (bottom panel) shows that also the low-frequency region is affected in the E242Q variant. Both the intensity of the vinyl binding modes at 329 and 410 cm^{-1} and the intensity of the ν_7 mode (678 cm^{-1}) have greatly increased.

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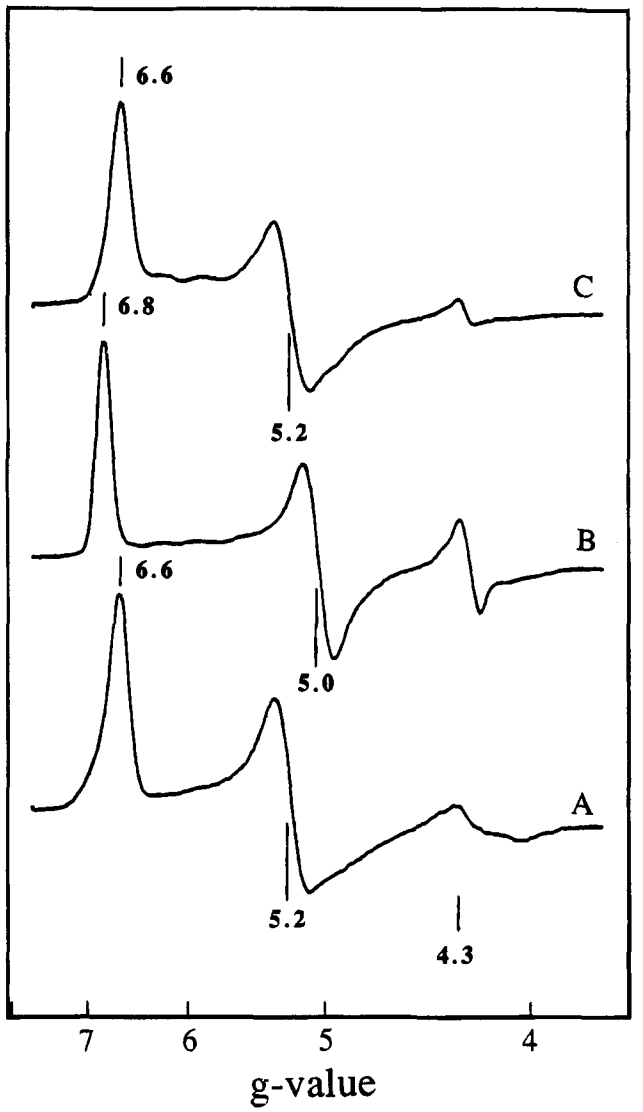

Figure 2. EPR spectra of native, recombinant, and E242Q myeloperoxidases: (A) native myeloperoxidase (15 μM) in 200 mM sodium phosphate (pH 7.2), (B) recombinant myeloperoxidase (15 μM) in 50 mM sodium phosphate (pH 7.2) and 300 mM chloride, and (C) E242Q myeloperoxidase variant (18 μM) in 100 mM sodium phosphate (pH 7.2). Conditions during the recording of the spectra were as follows: temperature, 12 K; frequency, 9418.6 MHz; modulation amplitude, 10 mT; microwave power incident to the cavity, 26 mW; gain 2.0×10^5 (E242Q), 1.25×10^5 (recombinant myeloperoxidase), 2.5×10^5 (native myeloperoxidase).

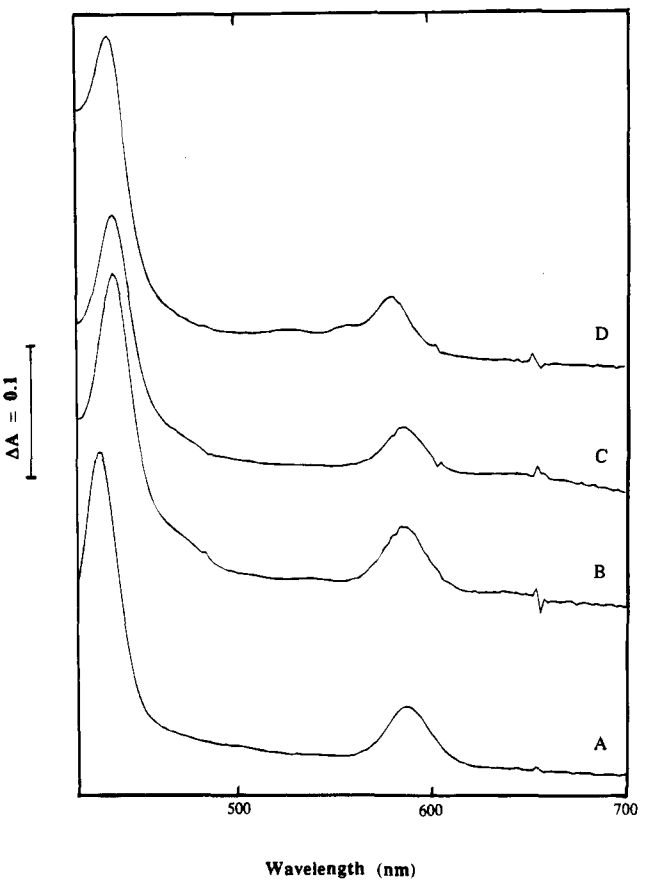

Figure 3. Alkaline pyridine hemochrome spectra of cytochrome *c* oxidase and native, recombinant and E242Q myeloperoxidases: (A) cytochrome *c* oxidase (1.2 μM), (B) native myeloperoxidase (1.2 μM), (c) recombinant myeloperoxidase (1 μM), and (D) E242Q myeloperoxidase (1.2 μM). The alkaline pyridine hemochrome spectra were prepared in 2.1 M pyridine and 75 mM NaOH, and a few grains of dithionite were added for reduction.

Myeloperoxidase is a rather unique enzyme since it is able to peroxidize chloride to hypochlorous acid.[3-5] It was shown that recombinant myeloperoxidase is also able to peroxidize chloride to hypochlorous acid, and the pH optimum was shown to be similar to that of native myeloperoxidase.[33] The chlorinating activity of recombinant myeloperoxidase at chloride concentrations between 50 and 200 mM was studied, and the Lineweaver–Burk plot indicates that chloride is a competitive inhibitor with respect to hydrogen peroxide as was found[5] for the native enzyme (not shown). The apparent K_m for hydrogen peroxide at pH 4.5 was calculated to be 1 mM. After correction for the inhibition by chloride, a K_m for hydrogen peroxide of 30 μM and an inhibition constant for chloride (K_i) of 5 mM were obtained. These values are similar to those reported for the native enzyme.[5] The E242Q mutant was not able to peroxidize chloride to hypochlorous acid, and this demonstrates that Glu242 plays an important role in this process. Guaiacol oxidation, however, was still observed, and the optimal pH of the reaction (pH 8.0 at 40 μM H_2O_2) was the same as that observed for the native enzyme. However, the peroxidatic activity of the mutant was about a factor of 10 lower (not shown).

Discussion

Upon exposure of myeloperoxidase to the denaturant guanidine-HCl or upon lowering of the pH, a reversible conversion of the rhombic high-spin EPR signal into a more axial high-spin signal was observed.[27,28] This phenomenon appeared to correlate to the blue-shift observed in the optical absorbance spectrum during these treatments and suggested a correlation between the blue-shift in the optical spectrum and the decrease in rhombicity of the EPR signal. However, the EPR spectrum of the E242Q variant of myeloperoxidase is typical for a rhombic high-spin heme system with *g*-values similar to those of native and recombinant myeloperoxidase. Thus, although the Glu242 to Gln mutation leads to a blue-shift in the optical spectra, the EPR spectrum is essentially unaffected, showing that there is no correlation between these spectroscopic properties. Probably there are additional interactions that cause the conversion of a high-spin rhombic EPR spectrum into a high-spin axial spectrum on both exposing the enzyme to guanidine-HCl and upon lowering the pH.

The exact chemical structure of the prosthetic group of myeloperoxidase is still not clear even though the crystal structure of the dimeric native enzyme is available.[31] We have shown that replacing Glu242 by Gln has a major effect on the optical spectrum of myeloperoxidase. However, the Soret band of the reduced mutated enzyme is still found at the unusual position of 458 nm (Figure 1) in agreement with Jacquet et al.[34]

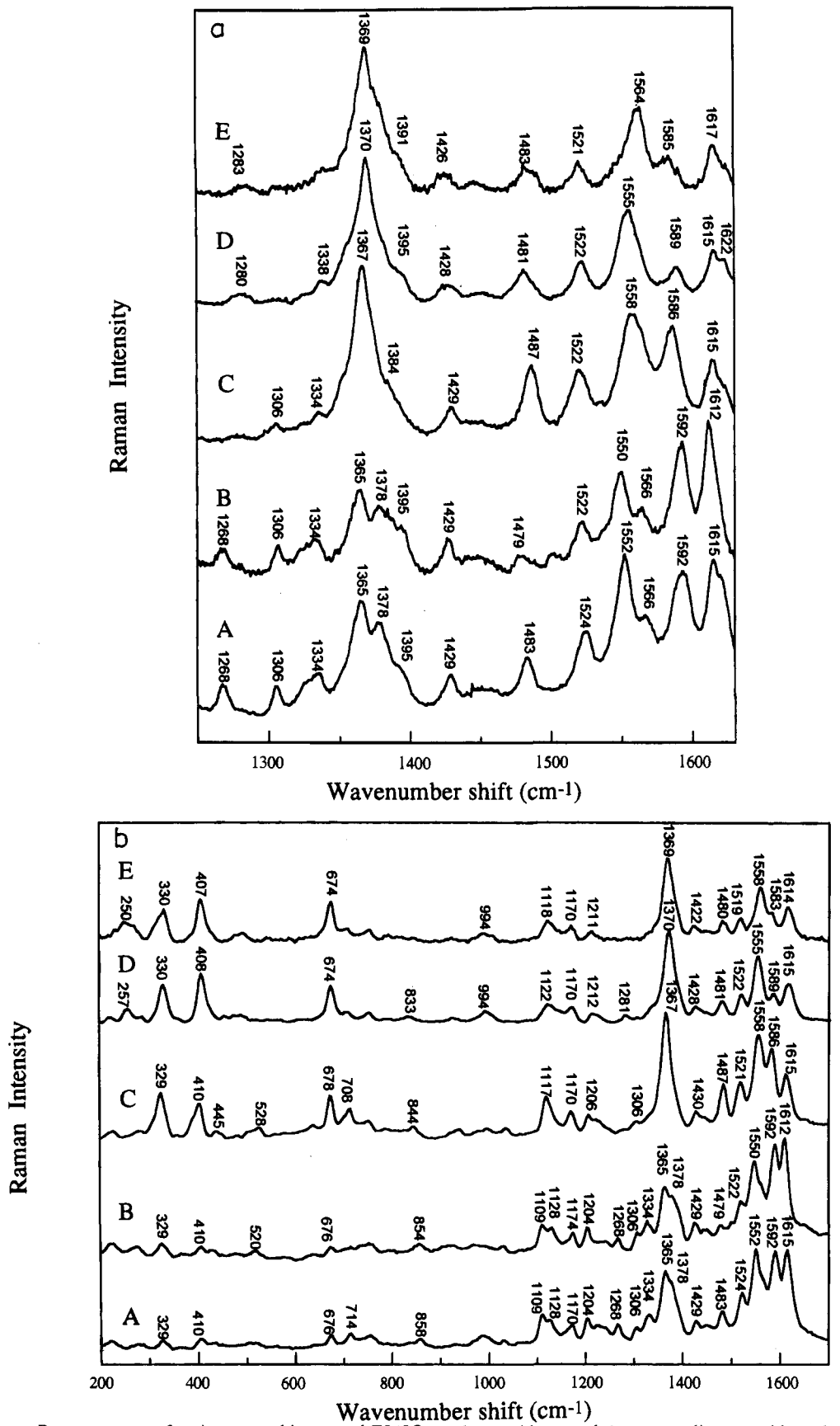

Figure 4. Resonance Raman spectra of native, recombinant, and E242Q myeloperoxidases and the mammalian peroxidases lactoperoxidase and eosinophil peroxidase. Top panel: (A) native myeloperoxidase (44 μM), (B) recombinant myeloperoxidase (30 μM), (C) E242 Q myeloperoxidase variant (50 μM), (D) bovine lactoperoxidase (77 μM), and (E) eosinophil peroxidase *in situ*. Laser power on the sample was 0.4 mW. The signal integration times were as follows: A, 300 s; B, 1000 s; C, 800 s; D, 300 s; spectrum E was averaged over 20 measurements on different cells. Resolution of the spectra is about 1 cm^{-1}. Bottom panel: (A) native myeloperoxidase (44 μM), (B) recombinant myeloperoxidase (30 μM), (C) E242Q myeloperoxidase variant (50 μM), (D) bovine lactoperoxidase (77 μM), (E) eosinophil peroxidase *in situ*. Signal integration time was 10 s/measurement. Spectra were averaged over 16 measurements. Samples A–D were in 200 mM sodium phosphate buffer (pH 7.2). The resonance Raman spectra were obtained with 413.1 nm excitation. Signal integration times for A–C were 300 s, and for D, 600 s. Laser power on sample was 0.4 mW. Resolution of the spectra is about 7 cm^{-1}.

and that of the denatured species is still at 448 nm.[27,28] This means that at least two factors contribute to the peculiar optical absorption spectra. On the basis of the pyridine hemochrome spectrum, it has been suggested[9,12,14-15] that an electron-withdrawing substituent such as a formyl group, like in heme a, is present on the heme periphery, which affects the electronic structure of the heme and is responsible for these optical absorption spectra. The pyridine hemochrome spectrum of the E242Q variant is very similar to that of native myeloperoxidase. This shows that the mutation in the protein backbone does not affect the chemical nature of the prosthetic group and demonstrates also that Glu242 is not involved in a covalent interaction with the chromophore in line with the suggestion made by Taylor *et al.*[45] This is in contrast to Zeng and Fenna,[31] who suggested on basis of the continuous electron density of Glu242 and the chromophore that this residue is esterified to a pyrrole nucleus of the prosthetic group, resulting in a covalent linkage. Taylor *et al.*[45] suggested that Met243 in fact links the prosthetic group to the protein, resulting in a chromophore with unusual optical spectra. We favor this proposal. It requires, however, that the pyridine hemochrome spectra of such a sulfonium derivative are similar to those of heme a, while the characteristic formyl resonance Raman frequency would be absent. A similar proposal of a thiol linkage has been made previously by Hurst.[46] It is interesting to note that in all mammalian peroxidases a Glu is conserved in the heme environment, but only in myeloperoxidase is a methionine (Met 243) present as a neighboring residue.[31] As pointed out, Met 243 may link the chromophore to the peptide chain and this may position the Glu242 in a special way different from that in other peroxidases. Unfortunately, X-ray crystal structures of other mammalian peroxidases are not yet available.

Resonance Raman studies on myeloperoxidase suggested that the prosthetic group of myeloperoxidase has a relatively low symmetry and is probably an iron chlorin.[16,23-26] The lowered effective symmetry of the prosthetic group in myeloperoxidase has a profound effect on the resonance Raman spectrum, which is rather complex, and especially in the oxidation-state marker (ν_4) region (1365 cm^{-1}), multiple lines arise due to symmetry reduction. The resonance Raman spectrum of recombinant myeloperoxidase is very similar to that of native myeloperoxidase with multiple lines in the ν_4 region (1365 cm^{-1}). Mutation of Glu242 to Gln has a drastic effect on the resonance Raman spectrum. In the oxidation-state marker region (ν_4) of the E242Q variant, a singlet line was observed at 1367 cm^{-1}, whereas in the resonance Raman spectra of both native myeloperoxidase and recombinant myeloperoxidase, multiple lines were detected. The resonance Raman spectrum of the E242Q variant is remarkably similar to those of the mammalian peroxidases lactoperoxidase and eosinophil peroxidase and is indicative of an iron protoporphyrin IX, which is the prosthetic group of both these enzymes. The only difference between the spectrum of the E242Q variant and those of both lactoperoxidase and eosinophil peroxidase is the intensity of the ν_{37} mode at 1586 cm^{-1}, which is higher in the spectrum of the E242Q variant. Photochemically modified myeloperoxidase[19] also displayed a symmetric resonance Raman spectrum but retained its chlorinating activity. In this case, the pyridine hemochrome spectrum of the enzyme species was affected due to chemical modification and it was concluded[19] that a peripheral substituent of the chromophore was modified. However, the nature of this modification is not known.

A notable feature in the resonance Raman spectrum of the E242Q variant is the higher relative intensity of the bands at 329 and 410 cm^{-1} as compared to the relative intensity of these bands in the spectra of native myeloperoxidase and recombinant myeloperoxidase. These bands result from the vinyl bending modes $\delta(CbC\alpha C\beta)$, which is in line with the observation that vinyl groups are present in myeloperoxidase.[19,23] In the spectra of both lactoperoxidase and eosinophil peroxidase these bands also have a higher relative intensity. A similar effect was observed for the ν_7 mode at 678 cm^{-1}, which also has increased in intensity in the spectrum of the E242Q variant as compared to both native myeloperoxidase and recombinant myeloperoxidase. The great similarity between the resonance Raman spectra of lactoperoxidase, eosinophil peroxidase, and the E242Q myeloperoxidase variant provides strong evidence in support of the suggestions made earlier[31,47] that the mammalian peroxidases are closely related. They contain virtually identical heme macrocycles but differ in heme–protein interactions.

Recombinant myeloperoxidase is able to peroxidize chloride to hypochlorous acid, and the Michaelis–Menten constant for H_2O_2, corrected for the inhibition by chloride, is very similar to that of native myeloperoxidase.[5] However, the E242Q variant of myeloperoxidase is not able to peroxidize chloride to hypochlorous acid, though it still acts as a peroxidase since it is able to peroxidize guaiacol and *o*-dianisidine.[34] This shows that the point mutation also greatly affects the catalytic properties of myeloperoxidase. Since the residue is located close to the macrocycle, it may be involved in creating further electronic asymmetry in the macrocycle to allow a more easy peroxidation of chloride during catalysis.

In conclusion, we have shown that Glu242 in myeloperoxidase has a major influence on the spectroscopic and catalytic properties of myeloperoxidase. The interaction of Glu242 with the prosthetic group is essential for the unique chlorinating activity of myeloperoxidase. Further, our results show clearly that interaction of the protein matrix (Glu242) with the chromophore leads to the apparent asymmetry in the electronic distribution, which in the past was interpreted to be the result of an iron-chlorin-like structure. This residue is also partly responsible for the red-shift in the optical absorbance spectrum of the enzyme, probably due to the presence of a negatively charged carboxylate group near the chromophore. It has already been suggested that the presence of the negative charge near a macrocycle may affect the absorbance spectra and lead to a red-shift of the absorbance maxima in the optical absorbance spectrum.[48] Additional protein–chromophore interactions must be present in myeloperoxidase that further affect the optical absorbance spectrum. We conclude that the prosthetic group of myeloperoxidase is an iron-protoporphyrin-like structure but with unusual spectroscopic properties similar to those of heme a. It may be that these spectral characteristics are imposed by a special sulfonium linkage with the Met243 as suggested by Taylor et al.[45] Site-directed mutagenesis studies of this residue will show whether this proposal is correct.

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