Essential Role of Proximal Histidine-Asparagine Interaction in Mammalian Peroxidases

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In heme enzymes belonging to the peroxidase-cyclooxygenase superfamily the proximal histidine is in close interaction with a fully conserved asparagine. The crystal structure of a mixture of glycoforms of myeloperoxidase (MPO) purified from granules of human leukocytes prompted us to revise the orientation of this asparagine and the protonation status of the proximal histidine. The data we present contrast with previous MPO structures and also, most strikingly, in the nature of the heme prosthetic group. Mature mammalian peroxidases are post-translationally modified with the heme covalently linked to the protein via autocatalytic formation of two ester bonds with highly conserved aspartate and glutamate residues (4–7). MPO is singular in having additionally a sulfonium ion linkage between the heme 1-vinyl group and a conserved methionine. The existence of these three covalent hemes to protein bonds has been correlated with the peculiar spectroscopic, redox, and catalytic properties of MPO (8, 9). Other, although more subtle, structural differences between the two heme peroxidase superfamilies concern the H-bonding partners of the essential proximal and distal histidines (4, 10).

Closely related with these structural peculiarities is the physiological role and the nature of the substrates of these oxidoreductases. MPO, EPO, and LPO are functionally homologous enzymes involved in host defense. Myeloperoxidase is secreted at inflammatory sites from stimulated polymorphonuclear leukocytes and also monocytes (11), whereas EPO is released from activated eosinophils (12). Lactoperoxidase is found in mucosal surfaces and exocrine secretions such as milk, tears, and saliva (13). In contrast, thyroid peroxidase is involved in the biosynthesis of the thyroid hormones thyroxine and triiodothyronine (14). These four metalloproteins prefer small anionic molecules as electron donors, such as halides (chloride, bromide, and iodide), thiocyanate, and nitrite (8). The corresponding oxidation products (e.g. hypohalous acids or nitrogen dioxide) are (strong) halogenating and nitrating oxidants that

The majority of currently known heme peroxidases, ubiquitous in all kingdoms of life, are members of two superfamilies that arose independently. The superfamily of (archae)bacterial, fungal, and plant heme peroxidases (sometimes called “non-animals” peroxidase superfamily) is represented by catalase-peroxidases, ascorbate peroxidases, cytochrome c peroxidases, manganese and lignin peroxidases, and plant secretory peroxidases (1, 2). The second superfamily (named the peroxidase-cyclooxygenase superfamily) was defined recently based on the reconstruction of the main evolutionary lines of mammalian heme peroxidases (3). This peroxidase-cyclooxygenase superfamily includes the mammalian peroxidases represented by myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), and thyroid peroxidase (3).

Both superfamilies differ greatly in their primary and tertiary structures and also, most strikingly, in the nature of the heme prosthetic group. Mature mammalian peroxidases are post-translationally modified with the heme covalently linked to the protein via autocatalytic formation of two ester bonds with highly conserved aspartate and glutamate residues (4–7). MPO is singular in having additionally a sulfonium ion linkage between the heme 1-vinyl group and a conserved methionine. The existence of these three covalent hemes to protein bonds has been correlated with the peculiar spectroscopic, redox, and catalytic properties of MPO (8, 9). Other, although more subtle, structural differences between the two heme peroxidase superfamilies concern the H-bonding partners of the essential proximal and distal histidines (4, 10).

The atomic coordinates and structure factors (code 3F9P) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The abbreviations used are: MPO, myeloperoxidase; LPO, lactoperoxidase; EPO, eosinophil peroxidase; HEK, human embryonic kidney; MCD, monoclonal iron-dedone; MD, molecular dynamics; PDB, Protein Data Bank.
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play an important role in the innate immune defense system but also contribute to tissue injury in certain inflammatory diseases (15). Due to their important role in (patho)physiology, these enzymes are of strong interest for the pharmaceutical industry (15).

X-ray structures of mammalian peroxidases have been published for one glycoform of canine (16) and human (4, 17) myeloperoxidase, as well as for caprine lactoperoxidase (5) (supplemental Table S1). Here, we present the crystal structure of a mixture of MPO glycoforms as obtained directly by purification from human leukocytes. The determined structure exhibits several significant differences compared with those reported in the literature. These variations include heme to protein linkages, sites, and extent of glycosylation as well as the interaction between proximal His\textsuperscript{336} and Asn\textsuperscript{421}. Specifically, the imidazole of His\textsuperscript{336} could be present as imidazolate. Its importance in maintaining the physical and catalytic properties of MPO is underlined by the fact that variant N421D exhibited modified spectral properties and completely lost its chlorination activity.

**EXPERIMENTAL PROCEDURES**

Materials—Buffy coat was kindly provided by the Department of Serology and Transfusion Medicine at the LKH, Graz, Austria. All chromatography columns and media were from Amersham Biosciences. Cetyltrimethylammonium bromide, phenylmethylsulfonyl fluoride, methyl-\textalpha\textbeta\textgamma-D-glucopyranoside, EDTA, sucrose, and all other chemicals were from Sigma and at the highest grade available.

Protein Purification—Human MPO was obtained from pooled buffy coat of about 200 different healthy donors. This MPO sample was a mixture of glycoforms and will be referred as hMPOm henceforth. In detail, buffy coat, the layer between plasma and red cells containing the leukocytes, was collected by centrifugation and washed twice with a solution of 155 mM NaCl, 40 mM sucrose, and 0.1 mM EDTA, containing hMPOm with a Soret maximum at 428 nm and a purity index (\(\frac{A_{428}}{A_{280}}\)) higher than 0.85 were pooled and desalted using Centriprep-30 concentrators (cut-off membrane, 30 kDa). Myeloperoxidase in 5 mM phosphate buffer, pH 7.0, was vacuum dried and stored at \(-80^\circ\)C.

**Crystallization and Structure Determination**—Vacuum dried hMPOm was solubilized with 50 mM Tris-HCl, pH 7.0, 100 mM NaCl up to a protein concentration of 10 mg/ml. Brownish crystals showing a tetragonal bipyramidal morphology were obtained at 20 °C by the vapor diffusion hanging drop method over a reservoir containing 10% polyethylene glycol 8000, 8% ethylene glycol, and 0.1 mM HEPES, pH 7.5. These crystals belonged to the tetragonal space group \(P_{4_1}2_12\) with unit cell parameters \(a = b = 110.7 \text{ Å} \) and \(c = 252.3 \text{ Å} \) and contained a dimer per asymmetric unit. A native data set was collected using synchrotron beam line ID14.4 at the European Synchrotron Radiation Facility from a crystal flash cooled using a cryo-protectant solution identical to its mother liquor but with increased ethylene glycol concentration (25%). Diffraction data were processed and scaled using the DENZO/SCALEPACK programs (18), respectively (see Table 1).

**TABLE 1**

| X-ray data collection and refinement statistics of hMPOm |
|---------------------------------------------------------|
| **Data collection statistics**                           |
| Space group                                             | \(P_{4_1}2_12\) |
| Unit cell parameters (Å)                                | \(a = b = 110.7, c = 255.3\) |
| Resolution (Å)                                          | 20–2.9 (3.1–2.9) |
| Unique reflections                                     | 33,805 (6,585) |
| Completeness (%)                                        | 96.0 (95.2) |
| \(R_{	ext{int}}\) (%)                                   | 9.7 (66.5) |
| \(\langle I/\langle I\rangle \rangle\)                  | 13.2 (2.7) |
| Redundancy                                              | 5.7 (4.9) |

| **Model refinement statistics**                          |
| Resolution                                              | 20–2.9 (3.0–2.9) |
| No. of reflections                                      | 31,861 (2,242) |
| Free reflections                                         | 1,707 (116) |
| \(R\) (Å)                                                | 23.6 (34.9) |
| \(\chi\) (%)                                             | 25.7 (34.8) |
| No. of residues                                         | 1,156 |
| Average \(B\)-factor (Å\(^2\))                          | 67 |

| **Ramachandran values**                                  |
| Preferred (%)                                            | 97.0 |
| Allowed (%)                                              | 2.8 |
| Outside (%)                                              | 0.2 (2 residues) |
Structure determination was achieved by molecular replacement with MOLREP (19), using as the search model the human MPO structure isoform C with PDB code 1CPX (4). Refinement, performed using REFMAC (20) and manual modeling with the graphic program O (21), gave final Rwork and Rfree agreement factors of 23.6 and 25.7%, respectively (Table 1). The reliability of the electron density maps allowed location of the main chain of all residues even if not present in PDB code 1CPX. Structure factors and coordinates are deposited in the Protein Data Bank (entry code 3F9P) with an imidazolate as the proximal iron ligand.

Molecular Dynamics Simulations—The starting MPO coordinates used in the molecular dynamics simulations were also derived from PDB 1CPX, as this is the human MPO x-ray structure at the highest resolution (1.8 Å) available in the PDB. Although, mature MPO is a dimer, the protomers (each containing a light and a heavy chain) work independently and exhibit spectral and catalytic properties indistinguishable from those of the dimer (10). Thus, for computational convenience, models were constructed with only one protomer, half of the MPO molecule (corresponding to chains A and C in PDB 1CPX). Sugars and acetate and sulfate ions were removed, whereas crystallographic water molecules, chloride, and calcium ions interacting with chains A and C were retained. Hydrogen atoms were added using standard bond lengths and bond angles. The N and C termini of the A and C chains were considered in their charged forms. All Arg, Lys, Asp, and Glu residues were considered in their ionized form except Asp94 protonated. The protein was solvated with ~22,000 water molecules and Cl– counterions were also included to neutralize the simulation cell. Differing in the protonation state of the proximal heme ligand His336, which also interacts with the amide group of Asp384 and a propionate (O–O 2.68 Å) suggested that Asp98 is protonated and this was supported by a preliminary MD analysis. Based on analysis of the local environment around each of the six histidines, all of them (except His336, see below) were considered in their ionized form except Asp98 protonated and His336 neutral. The AMBER (version 99) (22, 23) and TIP3P force fields (24) were used for simulation of the protein and water, respectively. Bonding and van der Waals parameters for the negatively charged His and for Met, Asp, and Glu covalently linked to the heme were provided by the GAFF extension of the AMBER force field (25). Point charges were derived according to the RESP procedure (26). Similarly, point charges were developed for heme-Fe(II). In the fitting, point charges on the porphyrin atoms were fixed at the AMBER heme-Fe(II) values and only the charges on the iron and the four pyrrole nitrogens were optimized.

Molecular dynamics simulations were performed with the NAMD program (27). Periodic boundary conditions were applied. The size of the simulation cell was ~102 × 87 × 88 Å. Long range electrostatic interactions were treated by the PME method. A 12-Å cutoff for the real part of the electrostatic and for van der Waals interactions was used. The integration time step was 1 fs. Bonds involving H atoms were kept rigid. After 1 ns of solvent equilibration and energy minimization of the whole system, each model was heated as follows: (i) 100 ps of NVT dynamics at 100 K, fixing the protein backbone; (ii) 100 ps of NPT dynamics (1 atmosphere, 100 K), fixing the protein backbone; (iii) 100 ps of NPT (1 atmosphere, 100 K); (iv) 100 ps NPT (1 atmosphere, 200 K); then, 2.5 ns of the MD simulations were performed at constant temperature (300 K) and pressure (1 atmosphere) (27).

Cloning, Expression, and Purification of Recombinant Wild-type MPO and the Variant N421D—Proteins were produced in two different mammalian cell lines, namely Chinese hamster ovary and HEK293. Transfection into hamster ovary cells, selection and culture procedures for transfected cells, as well as protein purification protocols were described in detail previously (28). Alternatively both wild-type and N421D were expressed in HEK293 cell lines. Briefly, full-length hMPO cDNA was introduced into pcDNA3.1(+) vector as the EcoRI-NotI cassette. For mutation of Asn421 a 1534 Kpn2I-NotI fragment was replaced by its mutated counterpart. The oligonucleotide primers used were: sense, 5′-CTGTGACATG TAGAGCAGGCAGG-3′ and antisense, 5′-GCATG(T/C) TAGAGCAGGCGAAGG-3′. After ligation and amplification in Escherichia coli JM107 and control sequencing, for transfection of HEK293 cells, standard selection and cultivation protocols were followed with minor modifications (29). Single stable producing colonies were selected for production in triple flasks (Invitrogen) for 4 weeks. Supernatant was collected every 2–3 days, passed over a 0.22-μm filter, and stored at 4 °C. Purification included cation exchange chromatography (Sephacryl CM fast flow; equilibration, 20 mM phosphate buffer, pH 7.5; gradient elution, 20 mM phosphate buffer, pH 7.5, 100–500 mM NaCl) followed by fraction pooling, sample concentration by ultrafiltration, and finally, size exclusion chromatography (Sephadex 200).

Circular Dichroism (CD) Spectrometry—Circular dichroism spectrometry was performed on a PiStar-180 (Applied Photophysics, Leatherhead, UK) spectrometer equipped with a thermostatic cell holder. The instrument was flushed with nitrogen with a flow rate of 5 liters min−1. For recording far UV spectra (180–260 nm) the quartz cuvette had a path length of 1 mm. Experimental conditions were: spectral band width, 5 nm; step size, 1 nm; scan time, 15 min; buffer, 5 mM phosphate buffer, pH 7.0, 25 °C. For comparison of secondary structure content both recombinant wild-type and N421D were brought to identical concentrations ascertained by identical absorption at 280 nm. Each spectrum was automatically corrected with the baseline to remove birefringence of the cell.

Enzyme Activity Assays—Enzymatic activity was determined polarographically and spectrophotometrically. Due to the fact that in the mutant protein heme occupancy was incomplete (see below), protein concentration was always calculated via heme absorbance to compare wild-type and mutant specific activities. Heme concentration was calculated via absorbance at the corresponding Soret maxima using an extinction coefficient of 91,000 m−1 cm−1 (9).

Consumption of hydrogen peroxide in the presence of halides was continuously monitored using a H2O2 electrode.
The platinum electrode was covered with a hydrophilic membrane and fitted to the Amperometric Biosensor Detector 3001 (Universal Sensors, Inc.). The applied electrode potential was 500 mV at pH 5.0 and the electrode filling solution was prepared fresh half-daily. The electrode was calibrated against known concentrations of H$_2$O$_2$. In detail, the consumption of 100 μM H$_2$O$_2$ in the presence or absence of 100 μM bromide or 100 mM chloride in 100 mM phosphate buffer, pH 5.0 (25 °C). The reaction was started by addition of 100 μM H$_2$O$_2$ and followed as absorbance decrease at 290 nm. Rates of halogenation were determined from the initial linear part of the time traces using an extinction coefficient for MCD of ε$_{290}$ = 19.9 mM$^{-1}$ cm$^{-1}$ (30). One unit of activity is defined as halogenation of 1 μmol of MCD per min at 25 °C.

Alternatively, halogenation and peroxidase activity were measured spectrophotometrically or fluorimetrically (Hitachi U-3000 and F-4500). The monochlorodimedone (MCD) assay was used to elucidate the chlorination and bromination activities (30). Experimental conditions used were: 100 μM MCD, 100 μM bromide, or 100 mM chloride, 50 mM wild-type MPO or N421D, 100 mM phosphate buffer, pH 5.0 (25 °C). The reaction was started by addition of 100 μM H$_2$O$_2$ and followed as absorbance decrease at 290 nm.

RESULTS

Structure of hMPOm—A mixture of MPO glycoforms (hMPOm) isolated from human leukocytes was crystallized as bipyramidal tetragonal crystals reaching about 0.5 mm in length. With a solvent content volume of about 55%, these crystals contained, in their asymmetric unit, a whole MPO molecule and diffracted up to 2.8-Å resolution. The structure was solved by molecular replacement using as the search model the coordinates from human MPO isoform C (PDB code 1CXP) (4). As expected the overall structure of hMPOm was very similar to the previously reported MPO structures (4, 16, 17) (Fig. 1 a).

The platinum electrode was covered with a hydrophilic membrane and fitted to the Amperometric Biosensor Detector 3001 (Universal Sensors, Inc.). The applied electrode potential was 500 mV at pH 5.0 and the electrode filling solution was prepared fresh half-daily. The electrode was calibrated against known concentrations of H$_2$O$_2$. In detail, the consumption of 100 μM H$_2$O$_2$ in the presence or absence of 100 μM bromide or 100 mM chloride in 100 mM phosphate buffer, pH 5.0, was followed at 25 °C. Reactions were started by addition of either 250 nM wild-type MPO or N421D. One unit of activity is defined as consumption of 1 μmol of H$_2$O$_2$ per min at 25 °C.

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For probing the peroxidase activity guaiacol or tyrosine were used as one-electron donors. Experimental conditions were: 100 μM guaiacol, 50 nM wild-type MPO or N421D, 100 mM phosphate buffer, pH 7.0 (25 °C). Rates of guaiacol oxidation were calculated by following the absorbance increase at 470 nm and using the extinction coefficient ε$_{470}$ = 26.6 mM$^{-1}$ cm$^{-1}$ (31). One unit of activity is defined as oxidation of 1 μmol of guaiacol per min at 25 °C.

Dityrosine formation from tyrosine (200 μM tyrosine, 100 mM H$_2$O$_2$, 50 nM enzyme, 100 mM phosphate buffer, pH 7, 25 °C) was followed spectrofluorimetrically (extinction wavelength, 325 nm; emission wavelength, 405 nm (32)).
due missing (4, 16, 17). The averaged root mean square deviation for the C/H9251 atoms with respect to the 1CXP structure is only 0.45 Å. Halide and calcium binding sites in hMPOm are mostly unchanged compared with the published MPO structures. However, in hMPOm, extra electron density was found close to Arg160 that could correspond to a chloride ion. Another chloride might be present in close proximity to main chain nitrogens around Cys152, enlarging the cluster of negative ions that bind along the molecular 2-fold axis. Additional electron density at Cys150 cannot be accounted for by a simple oxidation as proposed for the 1CXP structure (Fig. 1c).

The hMPOm structure appears to be extensively glycosylated (Fig. 1a). The MPO heavy chain sequence contains five potential asparagine glycosylation sites (Asn-X-Ser and Asn-X-Thr) and four (Asn157, Asn189, Asn225, and Asn317) had been biochemically confirmed (10, 34). However, in the 1CXP structure (4) only three N-glycosylation sites, with a total of eight monosaccharide moieties, were reported: Asn189 and Asn225 with a 1–4-linked N-acetylglucosamine and Asn317 with a branched (GlcNac)2-(Man)3 structure that has a fucose 1–6-linked to the first GlcNac. In the hMPOm structure the electron density indicates the presence of glycosylation also at Asn157 (Fig. 1d). At least two additional sugar monomers are visible at Asn189 and Asn317, which increases considerably the amount of sugars at the interface between the two halves of the dimer.

In animal heme peroxidases the prosthetic group is covalently linked with the protein. Published structures of MPO (4, 16, 17) demonstrated the existence of three covalent bonds. In hMPOm the ester bond between Asp94 and a hydroxymethyl group on pyrrole ring C is present, as is the sulfonium ion linkage between the \( \text{C/H9268} \) carbon of the vinyl group on pyrrole ring A and the sulfur atom of Met243 (Fig. 2a).

However, surprisingly, the side chain of Glu242 had a low electron density indicating high mobility and suggesting that the ester bond is only present in a fraction of the population of protein molecules (Fig. 2b). The electron density at the methyl-substituent of pyrrole ring A suggests its
Arg333 (hereafter referred as orientation OArg). In fact, a few of now between the amine group of Asn421 and the N atom of His336 (Fig. 2). Arginine 333 also interacts with the heme propionic group, 1 Å, leading (see below) to a strong repulsive interaction (Fig. 2).

FIGURE 3. MD simulations for two different protonation states of His336. In both cases the simulation starts with the OHis orientation: neutral His336 (a) and negatively charged His336 (b). The relevant distances among Arg333, Asn421 and His336, Asn421 are given. c, changes of the Asn421 orientation (defined by the time evolution of the Ω dihedral angle) during simulation for the two models considered: green line for neutral His336 and blue line for negatively charged His336.

hydroxylation, and the heme, although still considerably distorted, appears to be flatter compared with the published structures.

Special care had to be taken while modeling some of the distinct features of mammalian peroxidases in the heme proximal side (Fig. 2c). All previous MPO structures show the carbonyl group of Asn421 oriented toward the proximal ligand His336 (hereafter referred as orientation OHis) (supplemental Table S1). However, this places the amine group of Asn421 very close to the guanidinium group of Arg333, with a H–H distance <1 Å, leading (see below) to a strong repulsive interaction (Fig. 2d). Arginine 333 also interacts with the heme propionic group, which is expected to raise even further the pKₐ of the guanidinium group, making neutral (unprotonated) arginine very unlikely. Therefore, in hMPOm we considered flipping the amide group of Asn421 pointing its carbonyl group toward Arg333 (hereafter referred as orientation OArg). In fact, a few of the LPO structures recently deposited in the PDB already have the proximal asparagine in the OArg orientation (supplemental Fig. S1). The closest distances between the pairs Arg333-Asn421 and His336-Asn421 (2.80 and 2.86 Å, respectively) are in better agreement with the experimental resolution x-ray structure (4). In the final structure, the Asn421 side chain is slightly displaced with respect to the experimental crystallographic data, so that the carbonyl oxygen makes a bifurcated hydrogen bond with Arg333 and His336 (Fig. 3a and supplemental Fig. S1), whereas the amino group moves away from His336. Such a bifurcated hydrogen bond is not present in the x-ray structure (4).

As the assignment of the Asn421 orientation from x-ray experiments is uncertain, we compared the distances from the amide terminal heavy atoms, irrespective of their nature (O or N), to Arg333 and His336 in the high resolution x-ray structure (4) with the resulting conformations from MD simulations (Fig. 3). The closest distances among non-hydrogen atoms of the pairs Arg333, Asn421 and His336-Asn421 (3.00 ± 0.19 and 3.76 ± 0.33, respectively) differ substantially from the experimental structure (2.89 and 2.86 Å, respectively). The amino group of Asn421 does not form hydrogen bonds and is surrounded by hydrophobic residues.

A second simulation was performed starting again with the OHis orientation for Asn421 (Fig. 3b), but assuming now a negatively charged His336 side chain (His–). In this simulation the ~180° rotation of Asn421 took place very rapidly reaching the OArg orientation that was then continuously sustained (Fig. 3, b and c, and supplemental Fig. S2). In the resulting structure both the carbonyl and amino groups of the Asn421 side chain are forming hydrogen bonds with Arg333 and His336, respectively (supplemental Fig. S1). The closest distances between the pairs Arg333-Asn421 and His336-Asn421 (2.80 ± 0.10 and 3.05 ± 0.14, respectively) are in better agreement with the experimental structure (2.89 and 2.86 Å, respectively) (see also supplemental Table S1) than the values obtained with a neutral His336. Therefore, the MD simulations clearly demonstrate (i) that the com-
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Biochemical Analysis of the MPO Variant N421D—To probe the role of Asn^{421} in MPO catalysis recombinant wild-type MPO and N421D were heterologously expressed in and purified from two different mammalian cell lines (Chinese hamster ovary and HEK293) without obvious differences regarding the spectral and kinetic properties of the corresponding recombinant wild-type and mutant proteins. Fig. 4 depicts the overlay of far UV CD spectra of wild-type MPO and N421D brought to identical absorbance at 280 nm.

FIGURE 4. Spectral features of recombinant wild-type MPO and variant N421D. Optical absorbance spectra of ferric high-spin recombinant MPO (black line) and variant N421D (gray line) heterologously expressed in HEK293 cells. Conditions used were: 100 mM phosphate buffer, pH 7.0. Note that MPO purified from human leukocytes (i.e. sample used for x-ray crystallography) has identical spectral features as recombinant wild-type MPO. The inset depicts the overlay of far UV CD spectra of wild-type MPO and N421D brought to identical absorbance at 280 nm.

Interestingly, N421D showed a blue-shifted Soret band at 412 nm with a slight shoulder at 428 nm. The spectral intensity of this shoulder changed slightly depending on the point in time of protein harvest from both mammalian cell cultures. The purity number (\(A_{280}/A_{220}\)) of the respective proteins were 0.5−0.62 (wild-type) and 0.18−0.29 (N421D), indicating incomplete heme occupancy in the mutant protein. Thus, to guarantee comparability of enzymatic properties the concentration of active enzyme had to be calculated via Soret absorbance as has been demonstrated recently with other mutants (36, 38, 39) and described under “Experimental Procedures”.

Far UV CD spectra (inset to Fig. 4) indicated that the overall structures of both the wild-type and mutant proteins are very similar suggesting that exchange of Asn^{421} did not induce major structural rearrangements. If conformational changes did occur, they must have been very localized and minimal and thus were undetected by CD.

Together with modified heme absorbance the enzymatic activity of N421D was decreased compared with the wild-type protein. The variant completely lost the chlorination activity, whereas it still exhibited, although diminished, bromination activity, as has been determined by both the MCD method (2.9 ± 0.5%) as well as polarographically (1.9 ± 0.7%) compared with wild-type MPO (100%; 5.6 units/mg (MCD) and 29.8 units/mg (\(H_2O_2\) electrode)). Similarly, the peroxidase activity of N421D was lowered. The specific activity with guaiacol and tyrosine was 14.8 ± 1.5 and 6.0 ± 1.1%, respectively, compared with wild-type MPO (100%; 2.3 units/mg (guaiacol)).

DISCUSSION

One of the important structural differences between the two major heme peroxidase superfamilies concern the interaction of the proximal histidine. In non-animal (plant-type) heme peroxidases (1, 2) the proximal histidine is hydrogen bonded with a fully conserved aspartate that acts as a hydrogen bond acceptor deprotonating the N\(^6\) position of the imidazole. The resulting imidazolate character is mainly responsible for the negative standard reduction potentials (\(E'_{0}\)) of the corresponding Fe(III)/Fe(II) couples, which are typically within the range of −200 to −300 mV. These \(E'_{0}\) values guarantee the stability of the ferric form that is competent in binding and, finally, reduction of hydrogen peroxide. The imidazolate character of the proximal histidine strengthens the iron-imidazolate bond and helps to stabilize the oxidoreductase in its higher oxidation (i.e. compounds I and II) states.

In peroxidases belonging to the peroxidase-cytochrome oxidase superfamily (3) the proximal histidine interacts with a fully conserved asparagine and, based on published x-ray structures, it was generally assumed that the N\(^6\) proton is H-bonded to the neutral amide oxygen of Asn^{421} (similar to globins, where the proximal His is H-bonded to a neutral backbone carbonyl group). Skepticism about this proximal architecture in mammalian peroxidases was first raised by resonance Raman studies in the low-frequency region (9) that give valuable information...
about the iron-imidazole bond strength. Resonance Raman data demonstrated that in both MPO and LPO the iron-imidazole stretch is at fairly high frequencies (9) suggesting a substantial imidazole character of the proximal histidine also in these heme enzymes. Now, with the help of the presented crystal structure of hMPOm, comprehensive and critical analysis of all published structures of mammalian peroxidases (supplemental Table S1) as well as performed molecular dynamics simulations, this apparent contradiction can be explained. The presented data suggest that in mammalian peroxidases the NH$_2$ group of Asn$^{233}$ (MPO numbering) is oriented toward the unprotonated N$_6$ of the proximal His, whereas its carbonyl group interacts with the (positively charged) guanidinium group of Arg$^{333}$. A neutral proximal histidyl imidazole would manifest unfavorable contacts. Instead, deprotonated His$^{336}$ will strengthen the iron-imidazole bond thereby supporting the resonance Raman investigations (9). In addition, anionic His will stabilize higher oxidation states, an effect that is very important in MPO because it is known to form (transiently) catalytically active redox intermediates with very positive reduction potentials. Myeloperoxidase exhibits anomalously high $E'_0$ values of the redox couples Fe(III)/Fe(II) (+5 mV (9)), compound I/compound II and compound I/Fe(III), respectively (9, 37). These peculiar redox properties, induced by the strong electron withdrawing property of the MPO, typical sulfonium ion linkage (39), are a precondition for its physiological role that involves hypochlorous acid production (8).

In LPO, which lacks the sulfonium ion bond, the midpoint potential ($E'_0$) of the Fe(III)/Fe(II) couple was determined to be $-190$ mV (40) similar to plant-type peroxidases, thus demonstrating a comparable (de)protonation status of the proximal histidine in LPO and plant-type peroxidases. This suggests that the orientation of the proximal Asn in LPO might also be OArg and this is supported by few of the LPO structures recently deposited in the PDB (supplemental Table S1).

The importance of the close His-Asn-Arg interaction in maintenance of both the proximal and distal heme cavity architecture is supported by the observed effects upon exchange of Asn by Asp. The MPO mutant N421D showed a lowered heme occupancy as well as altered modes of heme to protein linkages as suggested by the calculated $A_{Soret}/A_{280}$ ratio and the blue (10 nm)-shifted Soret band. In addition, both its halogenation and peroxidase activity were significantly decreased. Myeloperoxidase exhibits anomalously high $E'_0$ values of the redox couples Fe(III)/Fe(II) (+5 mV (9)), compound I/compound II and compound I/Fe(III), respectively (9, 37). These peculiar redox properties, induced by the strong electron withdrawing property of the MPO, typical sulfonium ion linkage (39), are a precondition for its physiological role that involves hypochlorous acid production (8).

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probably do not have distinct physiological roles. MPO is coreleased from these granules during phagocytosis together with many hydrolytic enzymes including glycosidases (41). Thus, it is reasonable to assume that MPO glycoforms are an artifact of the isolation procedure in vitro or are produced during phagocytosis in vivo.

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