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SITE-DIRECTED MUTAGENESIS OF HUMAN MYELOPEROXIDASE: FURTHER IDENTIFICATION OF RESIDUES INVOLVED IN CATALYTIC ACTIVITY AND HEME INTERACTION

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Evidence for the involvement of four spatially clustered residues, Asp260(94), His261(95), Glu408(242) and Met409(243), in catalytic and spectral properties of human myeloperoxidase was provided by the analysis of site-directed mutants wherein these amino acids have been substituted by asparagine, alanine, glutamine and glutamine respectively. Although none of the mutations prevented folding, heme incorporation or secretion of the enzyme from transfected Chinese Hamster Ovary cell lines, the Glu408(242) to Gln and the Met409(243) to Gln substitutions led to a full blue-shift of the Soret peak, whereas the Asp260(94) to Asn modification led to a partial blue-shift. On the other hand, His261(95)->Ala and Met409(243)->Gln mutants totally lost the typical peroxidasic activity of the enzyme, whereas the Asp260(94)->Asn mutant was only partially active. These results confirm that His261(95) is the distal histidine essential for the catalytic activity of the enzyme while Asp260(94), Met409(243) and Glu408(242) are necessary for maintaining the correct conformation of the active site and all four residues that interact closely with the periphery of the heme contribute to the unique spectral properties of the heme in MPO.

Polymorphonuclear leukocytes contain an enzyme, myeloperoxidase (MPO, EC 1.11.1.7), which generates hypochlorous acid, a potent oxidant, in the presence of halides and hydrogen peroxide [1]. MPO is a heme-containing tetrameric glycoprotein, composed of two light (10-15 KDa) and two heavy (57-60 KDa) chains. Each heavy chain carries a covalently bound ferric heme prosthetic group, essential for the enzymatic activity [2]. MPO exhibits a characteristic absorption spectrum with a Soret peak at 428-430 nm, which is shifted to the red as compared with other hemic proteins [3]. Human recombinant MPO has been expressed in Chinese Hamster Ovary cells (CHO) as an 84 KDa heme-containing

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precursor having physico-chemical properties very similar to those of the natural mature enzyme [4,5].

On the basis of structure function studies of cytochrome C peroxidase [6] and of a three-dimensional structure analysis of canine myeloperoxidase [7], it has been possible to predict amino acid residues important for heme binding and catalytic activity in human MPO. One of these residues, His502(336) was recently shown to constitute the proximal histidine involved in the binding to the iron centers of the molecule [8]. Results of the X-ray structure analysis of canine MPO indicated His261(95) as the distal histidine and identified three other residues, Asp 260(94), Glu408(242) and Met409(243) that appeared to interact closely with the periphery of the heme prosthetic group [7]. It was proposed that Glu408(242) could be involved in a covalent linkage between the heme and the large polypeptide chain. To establish the function of His261(95) in MPO and to further analyse the roles of Asp260(94), Glu408(242) and Met409(243), we have created and characterized the respective site-directed Ala, Asn, Gln and Gln mutants of the enzyme. The results are consistent with the assignment of His261(95) as the distal histidine, essential for catalytic activity. Possible structural implications for the observed spectral differences between these mutants and the native enzyme are discussed.

Materials and Methods

Transfection of recombinant plasmids into CHO cells, selection and culture procedures for transfected cells, protein purification protocols, electrophoretic analysis, Western blotting, ELISA and peroxidasic activity assays for recMPO have been detailed previously [4].

The substitution mutants of myeloperoxidase, His261(95)->Ala, Asp260(94)->Asn, Glu408(242)->Gln and Met409(243)->Gln, were produced by replacing, in the MPO coding cassette carried by plasmid pNIV2703 [4], a 395 bp *Mro*I-*Apa*I DNA fragment or a 178 bp *Apa*I-*Avr*II fragment by their respective mutated counterparts. Mutations were generated within these fragments by a combination of polymerase chain reactions [9] and overlap extensions, using sets of oligonucleotide primers carrying the modified codons (Fig. 2). Amplified fragments were sequenced using Sequenase version 2 (U.S. Biochemical Corp., USA).

Final recombinant plasmids, pNIV2712, pNIV2713, pNIV2714 and pNIV2718, were transfected into CHO cells and G418 resistant colonies were selected and expanded in 175 cm^2 Falcon flasks. Cell culture supernatant (1 liter) was collected for each transfected cell line and processed to purify the mutant protein species [4,8]. Visible spectra were recorded on a Cary 15 spectrophotometer. Denaturation of proteins was performed for 35 minutes in 5M guanidine-HCl; reduction of proteins was carried out by the addition of sodium dithionite. The presence of heme in recombinant proteins was determined by

measuring the absorbance at 428 nm. Oligonucleotides were synthesized on an Applied Biosystems synthesizer model 380A via the solid-phase phosphoramidite method [10]. The positions of amino acid residues are identified by two numbers: the first one refers to the position in the recombinant promyeloperoxidase molecule [11] and the second one, in brackets, to the position in mature natural myeloperoxidase [12].

Results

Four site-directed mutations have been introduced into the cDNA coding for human MPO. The first one substituted His261(95), a putatively important residue for catalysis [7], by alanine, a small non-polar amino acid. The three other mutations replaced amino acids Asp260(94), Glu408(242) and Met409(243), predicted to interact with the heme [7,13], by asparagine, glutamine and glutamine respectively. The vicinity of the four targetted amino acid residues in the 3D structure of myeloperoxidase is shown in Figure 1. The mutations were created by a combination of polymerase chain reactions and overlap extensions using oligonucleotides carrying the appropriate mismatches (Fig. 2). After reconstruction of the mutated MPO coding modules, recombinant plasmids, pNIV2712, pNIV2713, pNIV2714 and pNIV2718 respectively, were used to transfect CHO cells in culture. Stable cell lines, resistant to geneticin, were obtained and shown by ELISA [4] to secrete the recombinant proteins in the medium at levels of about 1 µg/ml/24h. Spent culture medium (1 liter) was used to purify

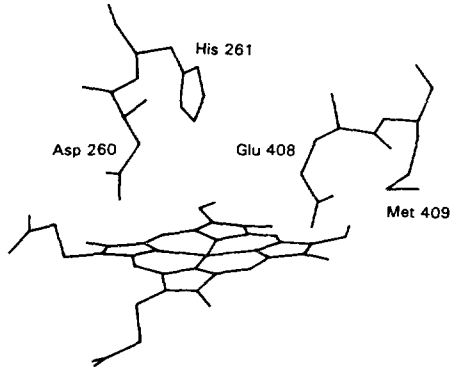

Figure 1.

Drawing of the protein environment on the distal side of the heme. Residues 260(94) and 261(95) in the H2 helix are shown together with residue 408(242) of the large polypeptide. A full explanation of the 3D modelling of MPO has been published before [7]. Numbers refer to the position in mature natural MPO; positions in recombinant promyeloperoxidase are between brackets.

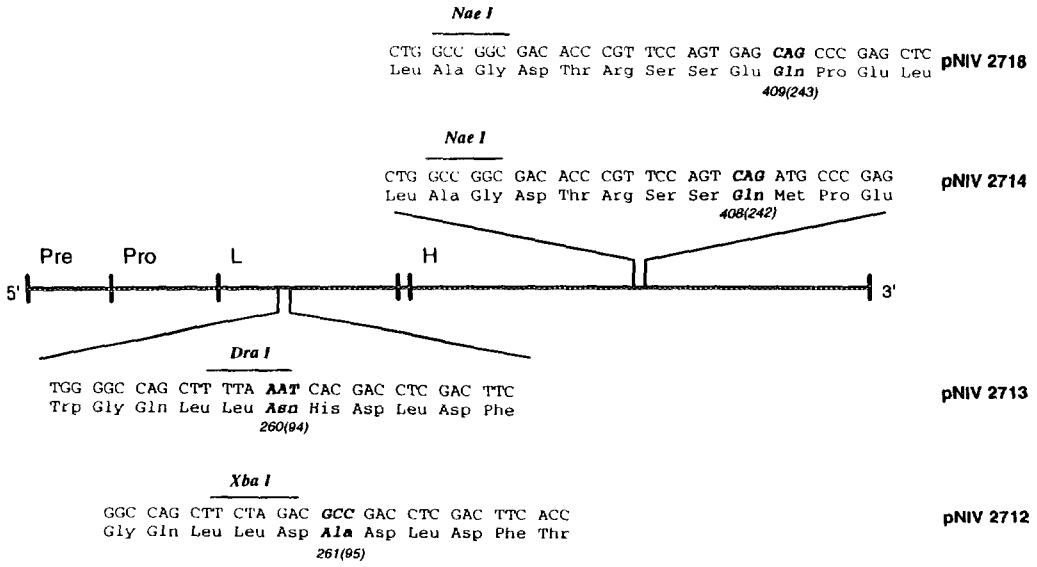

Figure 2.

Schematic representation of the site-directed mutagenesis of MPO

The full length (5' to 3') prepromyeloperoxidase cDNA is shown by the horizontal dark line; the signal peptide sequence, the prosequence and those for light and heavy chains are indicated by Pre, Pro, L and H, respectively. L and H are separated by 18 bp of DNA coding for 6 amino acid residues present in the recombinant MPO but absent from the mature enzyme (4). Synthetic oligonucleotides used for mutagenesis are shown with their translation. The mutated codons and the corresponding amino acids are in italic. Positions in recombinant and mature MPO are indicated below the relevant codon. Newly created restriction sites are indicated and underlined. pNIV2712, pNIV2713, pNIV2714 and pNIV2718, respectively, correspond to the His261(95)->Ala, Asp260(94)->Asn, Gln408(242)->Gln and Met409(243)->Gln mutant recombinant plasmids.

each of the mutant MPO molecules, according to the procedure described previously [4]; the overall purification yield obtained for each mutant species was about 70%. The purified proteins migrated on SDS-PAGE as single-chain immunoreactive precursors having identical apparent masses as those observed for the wild-type MPO, 84 kDa and 94 kDa [4, data not shown].

The specific peroxidasic activity of mutant MPO proteins was measured using O-dianisidine as substrate [14]. As seen in Table 1, MPO His261(95)->Ala was totally inactive in the assay, whereas MPO (Asp260(94)->Asn) was only partially active (21 U/mg) and MPO (Met409(243)->Gln) showed residual activity, as compared to the wild-type enzyme (108 U/mg). On the other hand, the mutation Glu408->Gln had no effect at all on the specific activity of MPO (112 U/mg). Visible spectra of the purified mutant proteins were then compared to that of

Table 1 Optical absorption maxima (nm) and specific activity (U/mg) for wild-type and mutant MPOs

Recombinant plasmid	Protein	Absorption maximum (nm)				Specific Activity
		Oxidized	Reduced	5M Gu-HCl oxidized	5M Gu-HCl reduced	U/mg
pNIV 2703	Wild-type recMPO	428 570	472 638	424 596	444 596	108
pNIV 2713	Asp260(94)→ Asn mutant	414 Shoulder 428 570	414 432 472 636	417 596	429 596	21
pNIV 2712	His261(95)→Ala mutant	415 Shoulder 428 570	n.d.	n.d.	n.d.	0
pNIV 2714	Glu408 (242)→ Gln mutant	416 556	458 572 624	n.d.	n.d.	112
pNIV 2718	Met409 (243)→ Gln mutant	412 514 640	443 560 640	n.d.	n.d.	2

Specific peroxidasic activity was measured using O-dianisidine as substrate (14).
Visible spectra were recorded on a Cary 15 spectrophotometer.
n.d.: not determined

the wild-type MPO species; the relevant absorption maxima, taken in various conditions, are listed in Table 1. The native mutant MPO His261(95)->Ala displayed the typical 570 nm band but, instead of having the Soret peak at 428 nm as is the case for the wild-type enzyme, presented a blue-shifted absorption maximum at 415 nm and a residual shoulder at 428 nm. The same phenomenon was observed for the mutant MPO Asp260(94)->Asn, in the native state, *i.e.* a blue-shifted Soret peak at 414 nm with a residual shoulder at 428 nm. Again, the position of the α-band at 570 nm was unaffected. In the reduced form, a shoulder appeared at 472 nm which corresponds to the normal wavelength for the absorbance maximum of reduced wild type MPO species. Further spectroscopic analysis of this mutant MPO, in reduced and oxidized state, also showed a partial blue-shifted position for the Soret peak without any difference in the position of the α-band as compared to the wild-type enzyme (Table 1).

In the case of mutant Glu408(242)->Gln, the absorption spectrum taken in oxidising conditions, revealed a single symetric Soret peak at 416 nm together with a 556 nm band. Upon reduction, these peaks were converted into 458 nm and

572 nm bands respectively. Whatever the conditions used, the absorption maxima remained clearly blue-shifted, compared to those for wild type MPO (472 nm and 638 nm). For the mutant Met409(243)->Gln, the absorption spectrum in oxidizing conditions showed a blue-shifted Soret peak at 412 nm and the α-band at 514 nm. The reduced spectrum of this mutant showed absorption maxima at 440 and 560 nm.

Discussion

X-ray crystal structures of 4 heme-containing peroxidases have now been determined. Yeast cytochrome C peroxidase [6] and a fungal lignin peroxidase [15] have similar three-dimensional structures but share only 18% sequence identity. Myeloperoxidase [7] and the catalytic domain of prostaglandin H synthase [16] are also very similar in three-dimensional structure despite their low level of 22% sequence identity. The structures of these mammalian peroxidases differ considerably from the fungal and yeast enzymes and their sequences show no significant levels of identity. In both myeloperoxidase and lactoperoxidase, the heme group is covalently bound to the polypeptide and unlike the hemes of yeast and fungal peroxidases, cannot be released by treatment with acidified acetone [17,18]. However, all 4 peroxidases possess a proximal histidine coordinated to the heme iron and a distal histidine that is required for peroxidase activity. Moreover, the distal and proximal histidines appear to be highly conserved in all mammalian peroxidases for which sequences are known [7,19]. In a previous paper [8], His502(366) was identified as the proximal histidine in agreement with the X-ray crystal structure. We have now shown that substitution of His261(95) by alanine completely abolishes the catalytic activity of the enzyme consistent with the X-ray crystal structure assignment of this residue as the distal histidine essential for peroxidase catalysis. The His261(95)->Ala mutant also produced a blue-shift in the Soret peak. This effect could be due to the loss of a bridging water molecule between the histidine and the iron atom of the heme. Similar blue-shifts of the Soret peak have already been observed for a species of myoglobin lacking the distal histidine and for a distal histidine mutant of cytochrome C peroxidase [20,21].

The Asp260->Asn mutant also exhibited reduced catalytic activity. Since this residue is close to the heme and also adjacent in sequence to the distal histidine, the reduction in the catalytic activity of this mutant may reflect a structural role for this residue in maintaining the correct conformation of the distal pocket rather than indicating any direct participation in the catalytic mechanism.

Since all three of the mutations Asp260(94)->Asn, Glu408(242)->Gln and Met409(243)->Gln exhibited absorption spectra with peak positions considerably blue-shifted with respect to those of native MPO, it is pertinent to consider the possible contributions of these residues to the unusual red-shifted spectrum of the native enzyme. It has been suggested that the red-shifted absorption in MPO could result from the presence of a negatively charged group such as a carboxylic residue in close proximity to the heme and that reversal of the red-shift upon denaturation of the enzyme with guanidine hydrochloride at low pH could be due to reprotonation of this carboxylic acid residue [13]. However, there is considerable evidence to suggest that the red-shifted absorption spectrum of MPO is not primarily attributable to a negatively charged carboxyl group on either of these two acidic residues in the native enzyme.

Comparison of the sequence of human MPO with those of the homologous mammalian peroxidases: eosinophil peroxidase (EPO), lactoperoxidase (LPO) and thyroid peroxidase (TPO) indicates that residues in close proximity to the heme in the X-ray crystal structure of MPO are highly conserved, with the notable exception of Met409(243) which is replaced by Thr in EPO, Gln in LPO and Val in TPO. Thus both Asp260(94) and Glu408(242) are conserved in mammalian peroxidases that have optical spectra which are not red-shifted but are more typical of other heme-containing proteins. Furthermore, the X-ray structure suggests that Glu408(242) is likely to be involved in a covalent link to the heme and would not therefore carry a negative charge in the native enzyme. On the other hand, Met409(243) which is a unique feature of the heme environment in MPO has recently been suggested to participate in a covalent bond to the heme via a possible sulfonium ion linkage [22]. This hypothesis is supported by a recent

high resolution (2.25 Å) X-ray crystal structure of human myeloperoxidase in which the sulfur atom of Met 409(243) has been shown to be within covalent bonding distance of the vinyl group on pyrrole ring A of the heme (R.E. Fenna, unpublished observation). It appears likely that this interaction could be primarily responsible for a difference in the spectral properties of MPO compared with other mammalian peroxidases that lack this methionine. However, the absence of catalytic activity for the Met409(243)->Gln mutant cannot be readily explained since the substituted amino acid glutamine occurs at this position in lactoperoxidase. If this methionine does form a covalent bond to the heme in MPO, the loss of catalytic activity could result from structural rearrangements that affect the relative orientations of the heme and other amino acid side chains in the distal pocket that participate directly in the catalytic mechanism.

The unusual spectral properties of the heme in MPO are presumably a consequence of a combination of interactions with the protein that are characterized by the unique participation of Met409(243) but also include the effects of Glu408(242) and Asp261(94), which also interact with the periphery of the heme in a manner that clearly exerts an effect on the spectral properties. In the absence of detailed structural information for these three mutants, the mechanism(s) by which their spectral properties have been altered from those of the native enzyme cannot, at the present time, be fully elucidated.

Acknowledgments

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References

1. Klebanoff, S.J. (1968) *J. Bacteriol. 95*, 2131-2138.
2. Andrew, P.C. & Krinsky, N.I. (1981) *J. Biol. Chem. 256*, 4211-4218.
3. Wever, R. & Plat, H. (1981) *Biochim. Biophys. Acta 661*, 235-239.
4. Moguilevsky, N., Garcia-Quintana, L., Jacquet, A., Tournay, C., Fabry, L., Piérard, L. & Bollen, A. (1991) *Eur. J. Biochem. 197*, 605-614.
5. Jacquet, A., Deby, C., Mathy, M., Moguilevsky, N., Deby-Dupont, G., G. Thirion, A., Goormaghtigh, E., Garcia-Quintana, L., Bollen, A. & Pincemail, J. (1991) *Arch. Biochem. Biophys. 291*, 132-138.
6. Finzel, B.C., Poulos, T.L. & Kraut, J. (1984) *J. Biol. Chem. 259*, 13027-13036.

7. Zeng, J. & Fenna, R.E. (1992) *J. Mol. Biol. 226*, 185-207.
8. Jacquet,A., Deleersnyder,V., Garcia-Quintana,L., Bollen,A. & Moguilevsky,N. (1992) *FEBS Lett. 302*, 189-191.
9. Higuchi,R. (1989) In: PCR Technology: Principles and Applications for DNA amplification (Erlich,H.A. ed.) Stockton Press, pp. 61-70.
10. Matteucci,M.D. & Caruthers,M.H. (1981) *J. Am. Chem. Soc. 103*, 3185-3191.
11. Johnson,K.R., Nauseef,W.M., Care,A., Wheelock,M.J., Shane,S., Hudson,S., Koeffler,H.P., Selsted,M., Miller,C. & Rovera,G. (1987) *Nucleic Acids Res. 15*, 2013-2028.
12. Iwamoto,H., Morita,Y., Kobayashi,T. & Hasegawa,E. (1988) *J. Biochem. 103*, 688-692.
13. Wever, R., Oertling, W.A., Hoogland, H., Bolscher, B.G.J.M., Kim, Y. & Babcock, G.T. (1991) *J. Biol. Chem. 266*, 24308-24313.
14. Krawisz, J.E., Sharon, P. & Stenson, W.F. (1984) *Gastroenterology 87*, 1344-1350.
15. Edwards, S.L., Raag, R., Wariishi, H., Gold, M.H. and Poulos, T.L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 750-754.
16. Picot,D., Loll, P.J. and Garavito,M. (1994) *Nature 209*, 243-249.
17. Schultz, J., Wu, N.C. and Marti, R. (1983) *Biochemistry 22* (15), 14A.
18. Morell, D.B. (153) *Australian J. Exptl. Biol. Med. Sci. 31*, 567-572.
19. Ortiz de Montellano, P.R. (1992) *Ann. Rev. Pharmacol. Toxicol. 32*, 89-107.
20. Shikama, K. & Matsuoka, A. (1989) *J. Mol. Biol. 209*, 489-491.
21. Smulevich, G., Miller, M.A., Kraut, J. & Spiro, T.G. (1991) *Biochemistry 30*, 9546-9558.
22. Taylor,K.L., Pohl,J. and Kinkade,Jr., J.M. (1992) *J. Biol. Chem. 267*, 25282-25288.