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Glu375Gln and Asp225Val Mutants: About the Nature of the Covalent Linkages Between Heme Group and Apo-Protein in Bovine Lactoperoxidase

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Abstract—In analogy with studies previously reported for myeloperoxidase (Kooter, I. M.; Moguilevsky, N.; Bollen, A.; Van der Veen, L. A.; Otto, C.; Dekker, H. L.; Wever, R. *J. Biol. Chem.* **1999**, *274*, 26794), we examined for bovine lactoperoxidase the effect of mutation of Asp225 and Glu375, the residues thought to be responsible for the covalent binding of the heme group to the apoprotein. Starting from the plasmid encoding rbLPO (Watanabe, S.; Varsalona, F.; Yoo, Y.; Guillaume, J. P.; Bollen, A.; Shimazaki, K.; Moguilevsky, N. *FEBS Letters* **1998**, *441*, 476), which was engineered to carry mutations in correspondence of those residues, the mutants Asp225Val and Glu375Gln were expressed in CHO cells and their products purified and characterized. Unequivocal evidence about the existence of ester linkages as well as their relative contribution to the specific spectroscopic and catalytic properties of bLPO is here discussed.

Peroxidases form a widespread and heterogeneous class of hemoproteins, showing a convergent catalytic mechanism. Their biological function is related to the oxidation of either organic or inorganic substrates,[1–5] by using H_2O_2 as co-substrate; for this reason peroxidases are considered non-specific enzymes. On the basis of differences in their primary, secondary and tertiary structures, they have been classified into two different super-families: plant, fungal and bacterial peroxidases, and animal peroxidases, respectively.[6] Myeloperoxidase (MPO), lactoperoxidase (LPO), eosinophil peroxidase (EPX) and thyroid peroxidase (TPO) constitute the subclass of mammalian peroxidases; they share a high sequence homology, which becomes superimposable among the active site-related residues.[7,8] MPO is the only member whose three-dimensional structure is available.[9,10] The prosthetic group was shown to be covalently bound to the apoprotein. Two ester linkages were described as provided by Glu408(242), Asp260(94) and two modified methyl groups on the pyrrole rings A and C respectively; a third linkage involved Met409(243) and a vinyl group of the heme. By combing both site-directed mutagenesis and spectroscopic analysis (i.e., EPR, UV–vis, NMR, FT-IR) the specific features of these linkages as well as their relative contributions to the heme binding were thus studied and accurately characterized. The heme group appeared strongly constrained, depending on the unique spectral and catalytic MPO properties.[11–15]

The lactoperoxidase crystal structure has not been resolved yet. Nevertheless, by using myeloperoxidase structure as template, an exhaustive theoretical model was obtained for both LPO and EPX.[16] In analogy to that claimed for MPO, the heme group of bovine LPO was proposed to be covalently linked to the apoprotein through two ester bonds involving Asp225, Glu375 and two hydroxymethyl groups on the porphyrin ring (Fig. 1).[16] The nature of these bonds in bLPO has been an object of interesting and long diversions. At first, Nichol et al.,[17] on the basis of both mass spectrometry and NMR characterization of the porphyrin macrocycle

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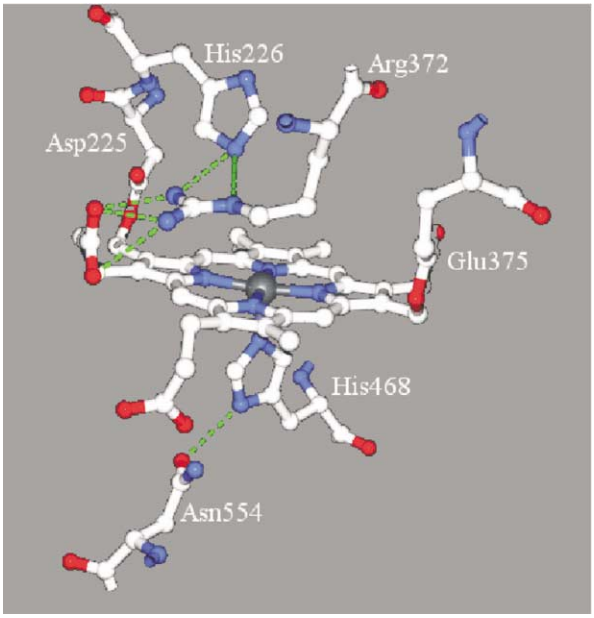

Figure 1. bLPO catalytic site, as described in the three-dimensional model.[16] All residues thought to play a crucial role in the catalytic mechanism are shown (namely His226, Arg372 and His468). The heme group appears covalently bound to the apoprotein through two ester linkages provided by Asp225 and Glu375. Green lines represent hypothetical hydrogen bonds.

upon cleavage of the enzyme under reductive conditions, reported a disulfide bridge at position 8 of the heme. This hypothesis was later denied, when further NMR[18] and spectral analysis[19] of the heme group strengthened the idea of ester bonds involving one or more positions of the porphyrin ring. This was in agreement with the predictions of the theoretical model; the only two cysteines not involved in disulfide bridges (Cys184 and Cys458) resulted one too far and the other not opportunely oriented with respect to the heme (steric hindrance) to form such linkages. In a recent communication, DePillis et al.[20] described the ability of bLPO to self-incorporate the heme group under an autocatalytic mechanism. By using a baculovirus expression system, a recombinant protein was produced, in which the porphyrin macrocycle was only partially bound to the apoprotein. It was shown that after incubating the purified apo-rbLPO with H_2O_2 and hemin, both modification of the heme and its covalent attachment to the protein were achieved. Two monohydroxylated heme intermediates were identified, supporting the previous hypothesis of a diesterified prosthetic group in native bLPO. Recently, biochemical evidences for heme binding through esters linkages have been reported also for human eosinophil peroxidase (EPX).[21]

Although strong evidence for the existence of covalent linkages between apoprotein and prosthetic group in LPO have already been provided by biochemical and spectral studies, some aspects still need to be clarified. For that, in analogy with studies carried out for recombinant MPO and its mutants,[13–15] we found it extremely interesting to approach the problem by means of site-directed mutagenesis. Starting from the bLPO recombinant plasmid,[22] opportunely engineered to carry point mutations in correspondence of the two residues thought to be responsible for the ester bonds, the mutants Asp225Val and Glu375Gln were expressed in CHO cells and their products purified by both cationic-exchange and affinity chromatography. Unequivocal evidence of the existence of ester linkages is shown and discussed.

cDNA construction

Two different strategies were followed for the preparation of the mutant cDNAs. For the preparation of the Glu375Gln plasmid a 93bp adaptor carrying the mutation was inserted as an *Asp*718I-*Nhe*I cassette into the plasmid encoding the Gln376Met mutant.[22] On the contrary, the plasmid pNIV2727 encoding rbLPO[22] was used as template for the construction of the Asp225Val cDNA. Two specific oligonucleotide primers were designed and purchased from GIBCO-BRL (*1660S:* AA AAC AGG TCC CTG CTC TTC ATG CAG TGG GGT CAG ATT GTG **GTC**; *2668AS:* CCG TGG ATC TTG TGA GTG GGG CTG GGA AAA GCT TGT TGC GCA GCT). A 971 bp mutated fragment was amplified by PCR and cloned into PCRII™ vector (Invitrogen) for sequencing. For the amplification the Hot Start Method[23] was chosen and the following conditions were applied: denaturation at 95 °C for 1 min, annealing at 58 °C for 2 min and extension at 72 °C for 1 min and 30 s. Thirty cycles were performed, with the mixture at last held for 10 min at 72 °C. The obtained adaptor was ligated into the plasmid pNIV2727 as an *Eco*O109I$_{(1666)}$-*Hind*III$_{(2637)}$ cassette,[22] leading to the plasmid pNIV2734 encoding the Asp225Val mutant.

Cells and transfection

For the expression of the mutants, CHO (Chinese Hamster Ovary) cells DG44 *dhfr*$^-$ [24] were chosen. For both pNIV2733 (Glu375Gln) and pNIV2734 plasmids (Asp225Val), 25 μg of DNA was transfected by electropermeabilization[25] and the resulting cells were maintained in α-MEM (+) medium supplemented with 5% fetal bovine serum, 2-mM L-glutamine, 1% penicillin/streptomycin and 0.7 μM hemin. Neomycin selection was carried out for 2 weeks. Positive clones were isolated and tested either for protein expression by ELISA and immunoblotting test, or for enzyme activity with ABTS as substrate. Large-scale cell culture was carried on for 3 weeks in cell factory devices (Nunc). Supernatants were harvested twice per week (roughly 400 mL per time), centrifuged, passed over a 0.22 μm filter and stored at 4 °C until purification.

Protein purification

The protein purification was achieved by combining cationic-exchange and affinity chromatography. A carboxymethyl-Sepharose (CM) fast flow column (2.2×15 cm) and a Blue-Sepharose CL-6B (2.5×15 cm) column were used. Both columns were equilibrated with

20 mM phosphate buffer pH 7.5. 1.6 L of culture supernatant was diluted with an equal volume of distilled water and loaded into the CM-Sepharose column (5–10 mL/min flow rate). The column was connected to a Gradifrac interfaced to an UV-detector and washed with phosphate buffer 20 mM, pH 7.5. A NaCl step-gradient (0, 0.15 M, 1 M) was applied for the protein elution. Each eluted fraction was assayed for peroxidase activity and the positive ones collected and diluted 1:4 with distilled water. Roughly 200 mL of protein solution was obtained and loaded into the Blue-Sepharose column; elution was achieved by applying the same conditions as described above.

Optical spectra

Optical spectra were recorded by using a Kontron Uvikon 930 double-beam spectrophotometer, with 10 mm-path length cells. Cell compartment was equipped with magnetic stirrer and temperature control device; 1mL volume quartz cells (Kartell) were used. Spectra were recorded between 200 and 700 nm and the ratio of distinct absorptions A_{413nm}/A_{280nm} was calculated to estimate the protein purity.

Activity measurements

Activity tests were performed both in 0.025 M acetate buffer pH 5.5 and 0.1 M phosphate buffer pH 7.0, by using either ABTS (2.5 mM) or SCN^- (0.2 mM) (SIGMA) as substrates. 10–100 nM enzyme solution in the sample cell was mixed up with substrate solution. H_2O_2 (MERCK, 0.2 mM) was added and the measurement was started immediately after. The formation of the oxidation product was followed for 1 min at 414 nm ($\varepsilon = 36.0\,mM^{-1}\,cm^{-1}$[26]) for ABTS and 235 nm ($\varepsilon = 1.29\,mM^{-1}\,cm^{-1}$[27]) for SCN^-; the enzyme activity was calculated from the slope of the curve determined during the first 6 s of reaction. Activity was expressed as μmol product/min^{-1} (mg enzyme)$^{-1}$.

ELISA and immunoblotting

Both ELISA and Western blotting tests were performed as previously described by Watanabe et al.[22] For ELISA, the 1C3-2A monoclonal antibody was employed at 1:7500 dilution, an anti-mouse alkaline-phosphatase-labeled IgG (1/5000) and *p*-nitrophenyl-phosphatase as substrate. For Western blotting, the same monoclonal antibody was applied at 1:10,000 dilution.

In all the experiments carried out, recombinant bovine LPO [22] was used as control.

The decision of mutating Glu375 in Gln and Asp225 in Val was mainly made by taking into consideration what was previously reported for myeloperoxidase mutants,[12–15] in a similar study addressed to clarify whether or not the MPO heme group is covalently bound to the apoprotein through ester linkages. Stable expression products in that case, beside the existence of a high sequence homology between MPO and LPO, represented for us a good indication to follow a similar strategy.

Glu375Gln DNA in pcDNA3 plasmid vector (pNIV2733) was prepared taking advantage of the previous construction of the Gln376Met mutant,[22] the adaptor carrying the mutation being the only difference. The sequence was controlled only in correspondence of the insertion and preceded by an extensive restriction sites analysis of the DNA, to exclude any reading frame shift.

For the Asp225Val mutant the PCR approach was preferred. Sequencing revealed that a silent mutation had been incorporated at the position 1672 (**G**CT⇒**C**CT), which was responsible for the suppression of one of the *Sap*I restriction sites.

Both plasmids were transfected in CHO cells, in order to prepare stable clones for large scale protein production. After neomycin selection, roughly 20 clones were selected for each mutant and grown in α-MEM(+) complete medium. On the basis of both ELISA and activity tests, one clone for Glu375Gln mutant (3E3) and one for Asp225Val mutant (3B6) respectively were chosen for the following cell factory, which lasted three weeks.

Supernatants collected and stored at 4 °C were at last purified (1.6 L batches), by both cationic-exchange on a CM-Sepharose column and affinity chromatography on a Blue-Sepharose column. The CM elution profiles was consistent with one single very sharp peak, whose fractions were characterized by both peroxidase activity and green color. On the contrary, after Blue-Sepharose elution two peaks were detected, only the first of which showed intense peroxidase activity. Starting from 2.4 L of supernatant, roughly 4.0 mg of active protein were recovered for both mutants. Figures 2a and b report SDS-PAGE and Western blotting for both Glu375Gln and Asp225Val, in comparison to rbLPO.[22] For both mutants, two bands were detected in the SDS gel, but only one of them resulted immunoreactive (Fig. 2b): namely 90 kDa for Glu375Gln and 92 kDa for Asp225Val. The smaller bands stained in the SDS gel might derive either from degraded protein for which the antibody epitope is missing, or from contaminant due to uncompleted purification. It is interesting to notice that, unlike rbLPO for which as previously reported[22] two closer components stained in the Western blotting, both mutants were characterized by only one band (Fig. 2b). This remark together with slight MW differences observed for both mutants, might account for an altered process of protein maturation.

Activity tests were performed either using ABTS or SCN^- as substrates and in both 0.025 M acetate buffer pH 5.5 and 0.1 M phosphate buffer pH 7.0. Results are summarized in Table 1 and compared to those obtained for recombinant bovine LPO in the same experimental conditions.[22] In all cases, both mutants showed a lower activity than that of rbLPO. When SCN^- was used as substrate an almost complete loss of catalytic activity was observed (Table 1). Studies of the binding modality

of inorganic ions to bLPO, carried out by optical investigation and corroborated by computer-assisted docking simulations, had shown that SCN^- is able to approach the heme group on the distal side, and interact with a specific protonable residue of the apoprotein, close to the iron, which might be the distal histidine (His226).[28] It is tempting for us to propose that both mutations might have caused a change of the heme location, which in turn could have suppressed or weakened the binding site.

With ABTS, which as all organic substrates is supposed to bind the protein in a specific hydrophobic pocket at about 13 Å from the iron,[5] a still high activity was found, where the Asp225Val mutant resulted much more effective than the other.

With the aim of elucidating whether or not the heme position had been affected by the mutations, UV–visible spectra of both mutants were recorded (Fig. 3) and compared to that of the recombinant enzyme.[22]

It appears evident that the two mutations are not equivalent in terms of their effects on the spectral properties of the enzyme. While the Asp225Val mutant did not result in any significant modification of the spectrum (Soret band at 413 nm and a similar pattern at higher wavelengths), the Glu375Gln mutant brought about a blue-shift of the Soret band from 413 to 408 nm (RZ = 0.45) which became sharper; further, a prominent charge-transfer band appeared at 527 nm.

Recently, it was proposed that the hypothesized ester linkage taking place between a glutamic acid and the heme group in all mammalian peroxidases, might be responsible for the red-shift of the Soret band in this class of enzymes, if compared to that of peroxidases from plant and fungal origin (i.e., HRP, 403 nm).[19] Besides, a precedent study addressed to understanding the nature of covalent linkages in MPO by the means of site-directed mutagenesis, had shown that the most effective mutation was indeed that involving the Glu242.[13] Our results seem to agree with both hypotheses. The present study definitely confirmed that the heme group in lactoperoxidase is covalently bound to

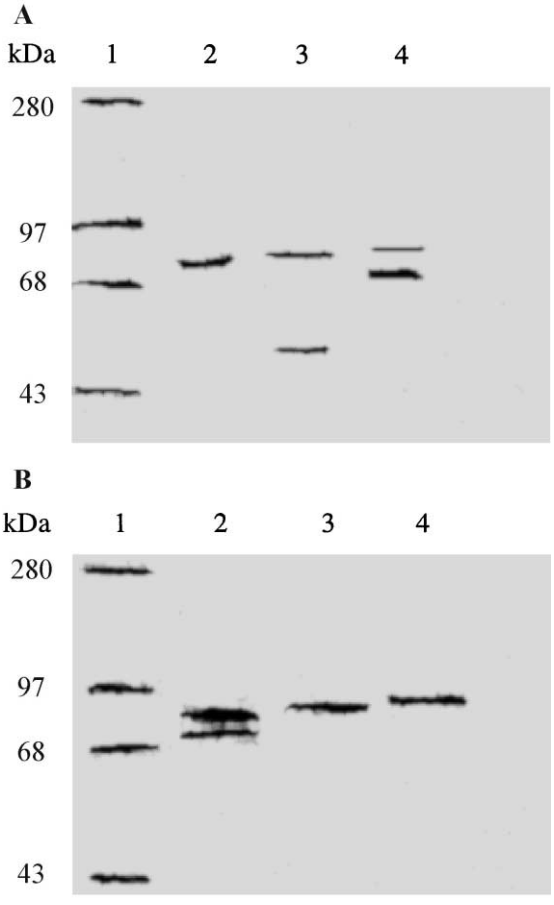

Figure 2. SDS-PAGE (a) and Western-blotting (b) 7.5% acrylamide gels for Glu375Gln and Asp225Val respectively, in comparison to those of rbLPO.[21] Ten μg of protein was loaded into the SDS gel, while 1 μg was used for immunoblotting; the monoclonal antibody IC3-A2 was used for immunoreactive detection. For both SDS and WB: Lane 1: molecular weight markers (280, 97, 68, 43 kD); lane 2: recombinant bLPO; lane 3: Glu375Gln; lane 4: Asp225Val.

Table 1. Specific activity calculated for Glu375Gln and Asp225Val and compared with that of rbLPO[22] in the same conditions. Either ABTS or SCN^- were used as substrates and measurements performed both at pH 5.5 in 0.025 M acetate buffer and pH 7.0 in 0.1 M phosphate buffer. Activity is reported as μmol product/min^{-1} (mg enzyme)$^{-1}$

Sample	SCN^- (pH = 5.5)	ABTS (pH = 5.5)	SCN^- (pH = 7.0)	ABTS (pH = 7.0)
rbLPO	230.6	464.6	66.9	112.7
Glu375Gln	5.5	12.8	0.9	25.1
Asp225Val	18.5	70.7	1.0	78.1

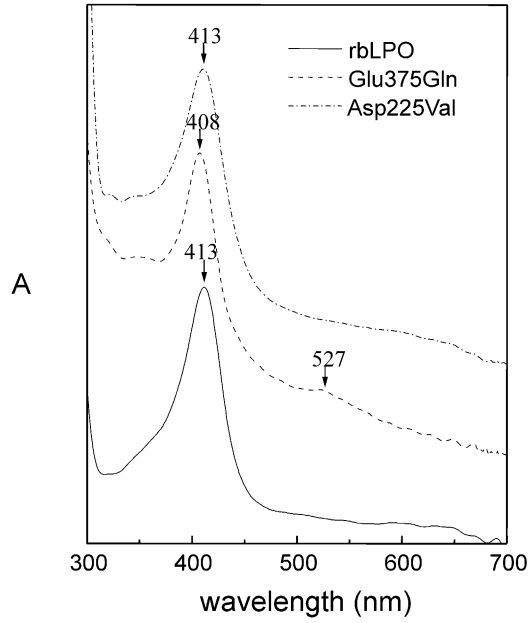

Figure 3. UV–vis spectra of rbLPO (——),[22] Glu375Gln (- - - -) and Asp225Val (–·–·–). The Asp225Val spectrum did not show significant modification with respect to the wild-type protein, the Soret band falling at 413 nm and the $RZ_{413/280}$ being 0.32; on the contrary the Glu375Gln showed its Soret band at 408 nm, for which the $RZ_{408/280}$ was 0.49.

the apoprotein through two ester linkages involving Asp225 and Glu375. Mutations able to suppress such linkages are translated in a lower level of heme incorporation; peroxidase activity appears negatively affected, especially towards SCN^-, which is supposed to bind the protein close to the heme group. The net change in the heme position, as a consequence of the introduced mutation, might affect the catalytic pocket conformation, with the loss (or disruption) of the substrate-binding site and in general a reduced accessibility to the protein core.

As already reported for MPO,[13] the two ester linkages appear to be not equivalent in lactoperoxidase. The loss of the Glu375 ester bond appears much more disruptive: as a consequence of the introduced mutation the LPO catalytic activity almost disappeared (either with ABTS or SCN^- as substrate) and a pronounced modification of the UV–visible spectrum took place, together with a net blue-shift of the Soret band. This is consistent with previous spectroscopic observations made on different mammalian peroxidases, which claimed the Glu residue as the responsible for the red-shift of the Soret band in comparison to that of fungal and plant peroxidases.[19]

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