Biochemical Evidence for Heme Linkage through Esters with Asp-93 and Glu-241 in Human Eosinophil Peroxidase

THE ESTER WITH ASP-93 IS ONLY PARTIALLY FORMED IN VIVO*

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The covalent heme attachment has been extensively studied by spectroscopic methods in myeloperoxidase and lactoperoxidase (LPO) but not in eosinophil peroxidase (EPO). We show that heme linkage to the heavy chain is invariably present, whereas heme linkage to the light chain of EPO is present in less than one-third of EPO molecules. Mass analysis of isolated heme bispeptides supports the hypothesis of a heme b linked through two esters to the polypeptide. Mass analysis of heme monopeptides reveals that >90% have a nonderivatized methyl group at the position of the light chain linkage. Apparently, an ester had not been formed during biosynthesis. The light chain linkage could be formed by incubation with hydrogen peroxide, in accordance with a recent hypothesis of autocatalytic heme attachment based on studies with LPO (DePillis, G. D., Ozaki, S., Kuo, J. M., Maltby, D. A., and Ortiz de Montellano P. R. (1997) J. Biol. Chem. 272, 8857–8860). By sequence analysis of isolated heme peptides after aminalysis, we unambiguously identified the acidic residues, Asp-93 of the light chain and Glu-241 of the heavy chain, that form esters with the heme group. This is the first biochemical support for ester linkage to two specific residues in eosinophil peroxidase. From a parallel study with LPO, we show that Asp-125 and Glu-275 are engaged in ester linkage. The species with a nonderivatized methyl group was not found among LPO peptides.

Known mammalian peroxidases, including myeloperoxidase (MPO)1 (1), eosinophil peroxidase (EPO) (2), lactoperoxidase (LPO) (3), and thyroid peroxidase (4), show 40–70% identity in pair-wise alignments. All enzymes contain a heme prosthetic group and use hydrogen peroxide as the electron acceptor in the catalysis of oxidative reactions. More distantly related members of this family, such as prostaglandin H synthase, have also been identified (5, 6).

MPO, EPO, and LPO are primarily found in granules of neutrophil and eosinophil leukocytes and secretions of exocrine glands, respectively. Their oxidation of halide and pseudohalide is part of the defense system against bacteria and parasites. Although several substrates have been found in vitro, the physiologically relevant substrates are believed to be chloride (MPO) and thiocyanate (EPO and LPO), which are oxidized to the toxic products hypochlorite and hypothiocyanite (7–9). Thyroid peroxidase functions in biosynthesis of thyroid hormones by oxidation of iodide (10).

LPO and thyroid peroxidase are single chain enzymes of about 75 and 100 kDa, respectively (10, 11). In contrast, the polypeptide chains of MPO and EPO are cleaved after synthesis to form a heavy chain of approximately 55 kDa and a light chain of approximately 15 kDa that remain associated. EPO is a monomer with the expected size of 70 kDa, whereas MPO is a disulfide-linked dimer of 150 kDa (12–16).

The mammalian peroxidases are distinguished from other heme-containing peroxidases by tight binding of the heme group to the apoprotein. Early experiments with LPO demonstrated that the heme group could not be extracted with acidic acetone and, furthermore, that it was not linked through thioethers as found in cytochromes. Based on this and other lines of evidence, heme linkage through ester(s) was proposed (17). But the heme group has also been suggested to be a thioderivative disulfide-linked to the protein (18). Results supporting and in conflict with both of these hypotheses followed (19–24).

Structurally, MPO is the best characterized of the mammalian peroxidases (5, 25). A 2.28-Å crystal structure has been obtained that allows evaluation of possible interactions between amino acid side chains of MPO and the heme group. Based on distances in the crystal structure, ester linkages from hydroxylated methyl groups on pyrrole rings A and C to Glu-242 in the heavy chain and to Asp-94 in the light chain, respectively, were proposed. It was further suggested that the vinyl groups of pyrrole ring A formed a sulfonium ion linkage with Met-243 (23, 25, 26). Unlike the two acidic residues, this methionine has no obvious equivalent in LPO or EPO (11, 25). It has also been suggested that Asp-94 and Glu-242 exist as protonatable residues capable of influencing the absorption spectrum of the heme group (27, 28). In short, however, after many years of investigation with mixed results, linkage of the heme group through two esters seems to be accepted for LPO and MPO, although for LPO, the residue that corresponds to Asp-94 in MPO has not been unambiguously identified (24).

Due to its limited availability, EPO is the least studied of the four peroxidases, and compared with LPO and MPO, insuffi-
cient evidence has been generated to allow generalization within the subfamily. EPO has primarily been the subject of comparative spectroscopic studies (23, 29–31), and no direct biochemical evidence has been reported.

Biochemical studies on mammalian peroxidases have previously been carried out on the isolated heme group as released with proteolytic enzymes, such as Pronase, that also cleaves ester bonds. Exceptions are recent spectroscopic studies on LPO (20, 24). In this work, we present data based on analysis of the isolated heme group with covalently bound EPO peptides. We chemically identify the bonds as esters, and we identify the residues that are engaged in this linkage. Furthermore, we demonstrate that in vivo one of the two esters is only partly formed but that autocatalysis in vitro results in formation of an enzyme with two esters. LPO was analyzed in parallel.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Human EPO was purified using a previously published procedure (32) with modifications. Briefly, isolated eosinophil granules from single donors with marked eosinophilia were extracted with 0.2 M sodium acetate, pH 4.0, and the supernatant was loaded onto a Sephadex G-50 (Amersham Pharmacia Biotech) equilibrated and eluted with 50 mM sodium acetate, pH 4.3. EPO eluted as a reasonably isolated peak. As judged by SDS-PAGE, the material was more than 90% pure (see Fig. 1). The original procedure included an additional ion-exchange chromatography step on CM-Sephadex in 0.1 M CAPS buffer, pH 10.0. In our hands, polymerization through cysteine residues is minimized to a negligible level if the pH is not elevated. For this study, nonpolymerized protein was important, and the purity obtained directly from the gel filtration column was sufficient. Therefore, the ion-exchange step was omitted. Different preparations of EPO were used. The functionality of EPO from patients with marked eosinophilia is the same as EPO purified before use by ion-exchange chromatography on a UNO-S12 column (Bio-Rad). The enzyme was dissolved in 35 mM sodium phosphate, pH 7.2. The flow rate was 0.2 ml/min, and the eluate was monitored by absorbance at 280 nm. The purity of the protein loaded in the column was assessed by SDS-PAGE and capillary electrophoresis. The A₂₈₀/₅₀₀ ratio of the purified protein was determined after addition of solid guanine hydrochloride to a final concentration of 6 M. Samples were separated by reversed-phase high pressure liquid chromatography on a 4 × 250-mm Nucleosil C18 column (Macherey-Nagel) eluted with a flow rate of 1 ml/min at 50 °C. A gradient was formed after making contact to the liquid and incubated for 20 h at room temperature prior to the chromatographic step was above 90%, we did not carry out further purification. Three bands appear in nonreduced SDS-PAGE of the preparation (Fig. 1, lane 3).

**RESULTS**

**Preparation and Characterization of Nonpolymerized Human EPO**—A preparation of human EPO that showed a minimum of polymerization through cysteine residues in nonreduced SDS-PAGE was used. In our hands, the traditional final step of ion-exchange chromatography at pH 10 (32) led to extensive polymerization of the protein. Because the purity of the protein prior to this chromatographic step was above 90%, we did not carry out further purification. Three bands appear in nonreduced SDS-PAGE of the preparation (Fig. 1, lane 2).
Recently, it was shown that covalent attachment of the heme group to the single polypeptide of LPO can occur by an autocatalytic process in the presence of hydrogen peroxide (22). With EPO, we have independently confirmed and visualized this process directly by SDS-PAGE (Fig. 1, lane 3). The intensity of the upper band increased with increasing concentrations of hydrogen peroxide (not shown). To bring >90% of the protein to the 70-kDa EPO form, a concentration of 30 μM hydrogen peroxide was required, corresponding to a 10-fold molar excess over EPO (Fig. 1, lane 3). Changing the pH in the reaction buffer to 6.0 or 8.5 did not affect the result. As expected, when the modified EPO was incubated with reductant, only heavy and light chain were seen in SDS-PAGE (not shown).

Isolation and Analysis of EPO Peptides with Covalently Bound Heme—Previously, indirect evidence for heme linkage through esters in the mammalian peroxidases, mainly LPO, was provided through investigations on isolated heme groups and by crystallography of MPO (25). Recent studies with LPO argue for ester linkage based on studies with isolated heme peptides, but complete assignment of amino acid residues could not be made (20, 24). In the general, direct peptide evidence has been lacking for all mammalian peroxidases.

EPO has not previously been investigated at the peptide level. To study the heme linkage in this protein and to study the basis for the apparently different attachment to the EPO light and heavy chains, peptides from a thermolytic digest were separated by reversed-phase high pressure liquid chromatography (Fig. 3A). The majority of peptides (90% based on peak heights) that show absorption at both 226 and 398 nm, thus
containing the heme group, were further analyzed.

N-terminal sequence analysis revealed that some were heme bispeptides (E-TL-2, E-TL-3), referred to below as bispeptides, and some were heme monopeptides (E-TL-1, E-TL-4, E-TL-5), referred to as monopeptides (Table I). All the bispeptides contained equimolar amounts of peptides derived from two regions in the EPO light and heavy chains, respectively. All monopeptides contained peptides derived from the heavy chain. This result, including the relative abundance of bispeptides (about 30%), is in fine agreement with the results from SDS-PAGE and gel filtration detailed above. Concordant results were obtained from several other digests with thermolysin, trypsin, and chymotrypsin alone or in different combinations (not shown and Table I). We never observed a monopeptide with polypeptide originating from the EPO light chain.

To substantiate the covalent heme linkages as esters and to identify the acidic residues putatively engaged herein, peptides E-TCT-101 and E-TL-3 (Table I) were immobilized on glass filters and incubated in an atmosphere of ammonia overnight at room temperature to convert the carboxyl moiety of the putative esters into amides by aminolysis. Sequence analysis of the peptides after incubation unambiguously identified the positions of heme linkage (Table II). In cycle 3 of peptide E-TCT-101 (239STETPK), partial conversion of Asp-241 to Gln was seen (not shown and Table I). The bispeptides contained peptides not treated with ammonia are given in parentheses.

A recent IUPAC recommendation for numbers of the heme b macrocycle differ from the numbering used in this study. In the recent IUPAC numbering (37), propionic acids are at positions 13 and 17; methyl groups at positions 2, 7, 12, and 18; vinyl groups at positions 3 and 8; and hydrogen atoms at positions 5, 10, 15, and 20. The orientation of the heme group is according to the orientation of the heme group in the x-ray structure of MPO (25).
The calculated average mass of nonderivatized heme b (C_{34}H_{32}FeN_{4}O_{4}) numbered from the N termini of the mature proteins (see Footnote 3).

Polypeptides of EPO and LPO.

Hydroxymethyl group at C-5, are shown in heme variants observed in monopeptides, having a methyl group or a hydroxymethyl group at C-1 and C-5. Here, heme b is shown with ester linkages between the Glu and Asp residues engaged in ester linkages (Table II). The two heme variants observed in monopeptides, having a methyl group or a hydroxymethyl group at C-5, are shown in a smaller font. Residues are numbered from the N termini of the mature proteins (see Footnote 3). The calculated average mass of nonderivatized heme b (C_{38}H_{34}FeN_{4}O_{4}) is 616.51 Da. The calculated mass of the expected heme contribution to the total mass in heme bispeptides (HC-C-1-heme b core-C-5-CH) is 4.03 mass units lower, i.e. 612.48 Da. This mass was observed for all EPO and LPO bispeptides analyzed (Table IV).

**Table IV**

| Peptide | Observed mass | Peptide mass | Nonpeptide mass |
|---------|---------------|--------------|-----------------|
| E-TL-1  | 1906.29       | 1275.38      | 630.91          |
| E-TL-2  | 2533.58       | 1921.06      | 612.52          |
| E-TL-3  | 2680.63       | 2068.23      | 612.40          |
| E-TL-4  | 1890.04       | 1275.38      | 614.66          |
| E-TL-5  | 2003.68       | 1358.54      | 615.14          |
| E-TCT-101 | 1276.33    | 661.71       | 614.62          |
| L-TL-1  | 1064.07       | 433.42       | 630.65          |
| L-TL-2  | 1643.65       | 1031.05      | 612.60          |
| L-TL-3  | 1567.73       | 736.78       | 630.95          |
| L-TL-4  | 2175.59       | 1562.65      | 612.94          |

* Observed mass minus peptide mass. The two variants of the heme monopeptides are mono-CH_{2}OH, which results from hydrolysis of the ester at C-5, and mono-CH_{3}, which originates from an EPO molecule where this ester had never been formed. Bis peptides, with both esters linked via esters, are shown.

**Discussion**

By analysis of intact human EPO and proteolytic peptides thereof, we show that 1) the heme group is covalently bound to both the light and heavy chain of the EPO polypeptide in less than one-third of EPO molecules, 2) the heme group is bound only to the heavy chain in the majority of EPO molecules, 3) incubation of EPO with excess hydrogen peroxide attaches the unbound EPO light chain to the heme group in an autocatalytic reaction, 4) the two acidic residues that are engaged in binding are Asp-93 and Glu-241, and as they can be converted into the corresponding amides by aminolysis, linkage by ester bonds is confirmed, and 5) in molecules where the heme group is attached to the heavy chain only, the site of possible light chain attachment is a nonderivatized methyl group that cannot result from ester hydrolysis. Parallel studies on LPO demonstrate that 6) the two acidic residues in the LPO polypeptide that bind the heme group are Asp-125 and Glu-241, and 7) both of these residues are bound to the heme group in the majority of LPO molecules. Our findings represent the first biochemical data on heme attachment in EPO, and our data on EPO and LPO are relevant to a long standing controversy on the nature of the heme attachment in mammalian peroxidases.

With isolated EPO bispeptides (Table I), we provide evidence for heme attachment through esters. First, the peptide masses are in perfect agreement with linkage of a heme b prothetic group by two esters (Table IV). Second, the bonds are susceptible to cleavage by ammonia (Table II). Concordant results were obtained with bispeptides derived from LPO (Tables III and IV). Previously, based on biophysical methods, ester bonds have been proposed for the heme linkage in MPO and LPO. Principally from a high resolution MPO crystal structure (25), and from spectroscopy of LPO peptides (24), two ester bonds were proposed. Recent infrared difference spectra of MPO, LPO, and EPO also point toward ester linkage (23).

The sites of heme attachment in EPO were unambiguously identified by sequence analysis of peptides converted by aminolysis as Asp-93 of the light and Glu-241 of the heavy chain (Table II), and in a similar experiment, the corresponding residues of LPO were identified as Asp-125 and Glu-275. This is the first biochemical identification of specific residues engaged in heme linkage for any of the peroxidases. Prior to this study, only LPO had been studied at the peptide level, and the peptides C_{38}H_{34}FeN_{4}O_{4} and C_{32}H_{32}FeN_{4}O_{4} could be released by alkaline hydrolysis from an LPO bispeptide (24). The first of those peptides suggests that Glu-275 is engaged in heme binding; the second leaves several candidate residues, including the Asp-125 identified here. With MPO, two candidate heme binding acidic residues were pointed out from the crystallographic structure of this protein, and for the first time convincing evidence for binding by esters was presented (25). Neither LPO nor EPO contains a residue equivalent to the methionine of MPO that is engaged in sulfonium ion linkage of the heme group (25). In conclusion, esters are common to heme linkage in MPO, LPO, and EPO.
Unreduced SDS-PAGE of EPO reveals that the majority of EPO exists in a form in which the light and heavy chain are not covalently linked (Fig. 1, lane 2). We are not aware of any publication that shows the result of unreduced SDS-PAGE of purified EPO. However, chain separation under these conditions was mentioned in one report (16). After incubation with reductant, the chain separation was complete (Fig. 1, lane 1), compatible with the earlier hypothesis, now rejected, of heme linkage by disulfide bond(s) (18). In denaturing gel filtration, the partial chain separation was also evident, and this experiment further demonstrated that the heme group is never bound to the EPO light chain alone (Fig. 2). After incubation with reductant, the protein chains are fully separated, and the heme group elutes separately (not shown). We conclude that the esters can be broken by incubation with reductant, possibly in a relatively rapid reaction of trans-esterification resulting in thioesters. However, the heme group itself also seems to be modified over time, but less rapidly, because its absorption at 398 nm decreased gradually in the presence of reductant. This might also be due to precipitation of the heme group.

Following incubation with hydrogen peroxide the protein appeared intact with both esters formed in SDS-PAGE (Fig. 1, lane 3). This experiment was prompted by an elegant, recent study (22) showing that heme attachment in LPO can occur by an autocatalytic reaction in the presence of hydrogen peroxide. With the two-chained EPO, this can be directly visualized in SDS-PAGE. Because the reaction product was still intact in unreduced SDS-PAGE, even under conditions of prolonged sample boiling, the chain separation described above was not a result of sample preparation. As expected, incubation with reductant rapidly caused complete chain separation (not shown).

A priori, the EPO monopeptides, invariably containing a peptide fragment derived from the heavy chain, would be expected to result from selective hydrolysis of the ester to the light chain. But of five thermolytic peptides from one digest (Fig. 3A and Table I), only one (E-TL-1, less than 10% abundance) has the expected mass of a peptide with a hydroxymethyl group at C-5 of the heme group. The other EPO monopeptides differ in mass by 16 units, corresponding to the presence of a nonderivatized methyl group on C-5 (Table IV, and Fig. 4). An obvious interpretation of this finding is that the ester to the light chain of EPO had never been formed during biosynthesis. Because a methyl group is known to function as a substrate in thioesterases, this result suggests that the heme group occurs more readily than at C-5, and possibly that esterification at C-5 requires that the ester at C-1 is already formed. But that does not explain why the limited ester hydrolysis seems to occur only at C-5 in both EPO and LPO. We speculate that the propionic acid at C-6 is able to break the Asp-esters of the hydrolytic equilibrium of this seven-member ring would greatly favor the hydroxy acid, which is the species we observed.

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