Resonance Raman characterization of the heme prosthetic group in eosinophil peroxidase

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Received 3 July 1985

The resonance-enhanced Raman spectrum of eosinophil peroxidase (EPO) from horse and human eosinophils is reported. Based upon the spectral energies, distribution and depolarization ratios of the high-frequency skeletal modes and upon the presence of weak bands assignable to vinyl substituent groups, we conclude that the heme prosthetic group is high-spin, 6 coordinate protoporphyrin. The Raman spectrum reveals clear differences from lactoperoxidase (LPO), an enzyme which appears nearly structurally isomorphous by other physical techniques; the data indicate a stronger axial 6th ligand in EPO. Mechanistic implications are discussed in relation to LPO and myeloperoxidase, an enzyme present in neutrophils and monocytes which contains a unique functional active-site chlorin.

Chlorin *Eosinophil* *Enzme mechanism* *Peroxidase* *Protoheme* *Raman spectroscopy*

1. INTRODUCTION

The neutrophil peroxidase, myeloperoxidase (MPO), is capable of catalyzing the two-electron peroxidation of the chloride ion to hypochlorous acid (HOCl) [1]. Several lines of evidence suggest that HOCl formed in this way is an important microbicidal agent in stimulated polymorphonuclear leukocytes [2,3].

The heme functional group of MPO is covalently bound to the protein and undergoes chemical modification upon attempts to isolate it [4]. This circumstance has hindered identification of the heme structure by conventional means. We have recently demonstrated by resonance-enhanced Raman spectroscopy that the heme of canine MPO is a chlorin [5]. Others have shown that the human enzyme gives a nearly identical resonance Raman spectrum and similarly concluded that its prosthetic group is a chlorin [6]. This assignment is consistent with physical properties of the enzyme measured by other techniques [7] but is at odds with chemical derivatization studies, which have suggested that the heme is a formylporphyrin [4].

The functional significance of an active site chlorin is unclear. In general, Compound I ferryl π-cations of porphyrin-containing peroxidases are incapable of oxidizing chloride ions, presumably because the energetics are unfavorable. Ring reduction to form the corresponding chlorins would be expected to decrease the Compound I potential by about 200 mV [8]; on thermodynamic grounds, therefore, chlorins are expected to be less effective catalysts of halide oxidation than structurally analogous porphyrins. We have suggested two possible ways in which chlorins might facilitate chloride ion oxidation [5]. Axial binding of the weak-field chloride ion to heme iron might be promoted in the MPO chlorin because the greater flexibility of hydroporphyrin rings brought about by disruption of ring π-conjugation permits relatively easy adjustment in core size, facilitating central metal binding of diverse ligands [9]. This explanation appears unlikely in view of recent

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00145793/85/$3.30

kinetic studies that identify the MPO heme chloride-binding site as inhibitory [10]. Alternatively, the presence of a chlorin Compound I may favor chloride ion oxidation by a peripheral ring position. The predominant reaction between chloride ion and metalloporphyrin π-cations [11] and other aromatic π-cations [12] appears to be electron transfer, rather than nucleophilic addition leading to ring chlorination. Reduction of HOCl is extraordinarily sensitive to the nucleophilic character of the reductant [13]. Assuming the reverse process, chloride ion oxidation, occurs through a transition state with similar properties, reactivity will be controlled by the electrophilic character of the oxidant electron acceptor site. As a consequence of having one of their pyrrole rings reduced, chlorin ferryl π-cations possess a ring charge asymmetry not found in analogous porphyrins [14]. This asymmetry might provide an unusually reactive electron-deficient peripheral site for chloride ion oxidation.

To test these postulated structure-function relationships, we use resonance Raman spectroscopy to identify the active-site heme group in eosinophil peroxidase (EPO). This enzyme is also capable of chloride ion peroxidation [15]. It has been proposed to contain active site iron protoporphyrin [16] based upon the resemblance of its optical and electron paramagnetic resonance spectra to those of lactoperoxidase (LPO), an enzyme known to contain protoporphyrin [17]. Resonance Raman spectroscopy permits assignment of spectral features to discrete functional groups of a chromophore, thereby providing a more definitive analysis of structure.

2. MATERIALS AND METHODS

Horse EPO was purified as described [18]. The final preparation, which was in 0.05 M sodium acetate buffer, pH 4.7, containing 1.0 M NaCl, had an A_{415}/A_{280} nm ratio of 1.05. The human EPO preparation was the void volume of a Sephadex G-50 chromatographic separation of solubilized human eosinophil granules from a patient with eosinophilia [19] (kindly provided by Dr Gerald Gleich). This preparation was in 0.05 M sodium acetate buffer, pH 4.3, containing 0.15 M NaCl. Based upon the molar extinction coefficient, $\epsilon_{413} = 110\ mM^{-1} \cdot cm^{-1}$ [16], the effective peroxidase concentration of the human preparation was 1.8×10^{-5} M; the measured absorbance ratio A_{413}/A_{275} was 0.38. Raman spectra were obtained for the suspensions using previously described procedures and instrumentation [5]; no evidence of sample degradation was observed in successive scans. Cyanide derivatives were prepared by spectrophotometric titration with solid potassium cyanide (Baker reagent grade) [5].

3. RESULTS

Chlorin resonance Raman spectra typically contain a relatively large number of bands [20], many of which are intense [21]. Raman spectra of horse or human EPO (figs 1 and 2) do not show these features, but instead are similar to known protoporphyrin-containing proteins, e.g. hemoglobin [22], intestinal peroxidase [23], horseradish peroxidase [24] and lactoperoxidase [25]. Further-

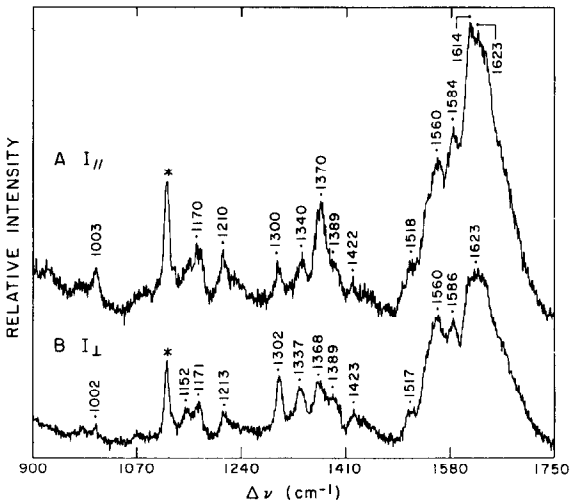

Fig.1. Resonance Raman spectra of EPO under 406.7 nm excitation, 90° scattering geometry, ~4°C, scan rate 1 $cm^{-1} \cdot s^{-1}$. Spectrum A: oxidized horse enzyme, 14 mW incident power, 6 scans. Spectrum B: oxidized human enzyme, 30 mW, 2 scans. Spectrum C: cyanide-derivative of human enzyme, 32 mW, 9 scans. Inset: oxidized human enzyme, low-frequency region, conditions as in B. Asterisk indicates laser plasma emission line. Oxidized human enzyme band frequencies and depolarization ratios ($I_{\perp}/I_{\parallel}$): 1613 (0.3); 1581 (0.3); 1557 (0.2); 1512 (0.2); 1477 (0.3); 1418 (0.7); 1365 (0.3); 1207 (0.3); 1167 (0.5); 1116 (0.3); 988 (0.5).

more, several anomalously polarized vibrational bands ($I_\perp/I_\parallel > 3/4$) are observed with excitation into the heme visible absorption bands. With 514.5 nm laser excitation, these bands are found at 1560, 1423, 1389, 1337 and 1302 cm^{-1} (fig.2). The appearance of anomalously polarized bands requires that the heme be a relatively high-symmetry porphyrin [26,27]. This excludes low-symmetry chlorin, which exhibits only polarized modes ($I_\perp/I_\parallel < 3/4$) [5,21].

Additional information regarding the porphyrin structure can be obtained from the Raman spectrum [28]. The positions of the oxidation state marker band (ν_4) at 1365 cm^{-1} in the resting enzyme (fig.1A,B) and at 1366 cm^{-1} in the cyano derivative (fig.1C) indicate that the central iron is ferric; the heme appears to be 6-coordinate, based upon a ν_{10} band frequency of 1614 cm^{-1} (fig.1A,B) [29]. The shift in the core-size marker band (ν_{11}) from 1557 to 1572 cm^{-1} accompanying cyanide addition indicates ligation occurs with high-to-low spin conversion of the ferric electronic configuration. The presence of vinyl substituent groups on the porphyrin ring can be inferred from weak bands at 1620 [$\nu_{C=C}(2)$], 1336 [$\delta_s(=CH_2)(2)$], 1304 [$\delta(CH=)$] and 1167 cm^{-1} [$\nu_{C_b-C_\alpha}(1)$] under Soret excitation (fig.1B), and at 1423 [$\delta_s(=CH_2)(1)$] and 1337 cm^{-1} [$\delta_s(=CH_2)(2)$] under visible excitation (fig.2B). Band assignments given in brackets correspond to those determined for metalloprotoheme complexes from frequency shifts accompanying deuteration [30,31].

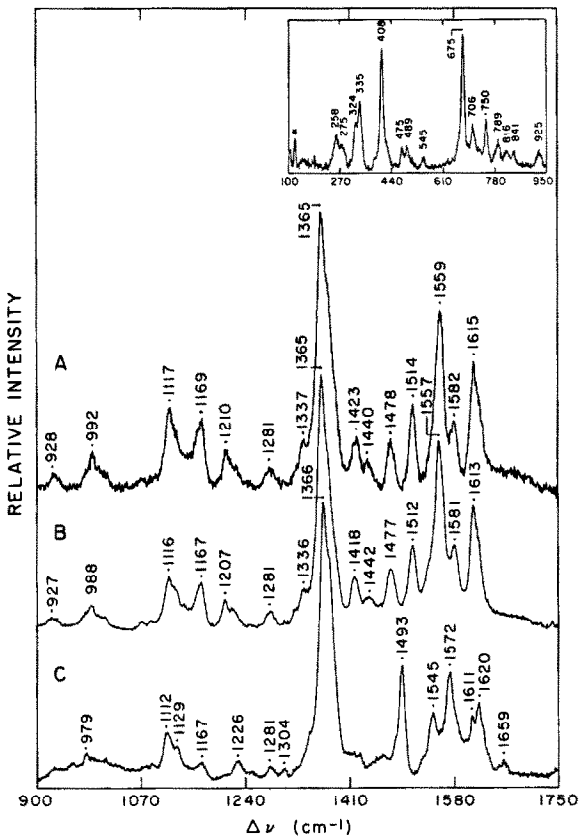

Fig.2. Resonance Raman spectra of human EPO under 514.5 nm excitation. Spectrum A: oxidized enzyme, parallel polarized light, ~4°C, 75 mW incident power, 17 scans, scan rate 1 $cm^{-1} \cdot s^{-1}$, 90° scattering geometry. Spectrum B: same as A except perpendicular polarized light. Asterisk indicates an emission line (435.8 nm) of the external fluorescent room lighting. Band frequencies and depolarization ratios ($I_\perp/I_\parallel$): 1623 (0.6); 1422 (1.3); 1389 (1.0); 1370 (0.4); 1340 (1.0); 1300 (1.2); 1210 (0.4); 1170 (0.6); 1003 (0.3).

4. DISCUSSION

The Raman spectra presented here suggest that the prosthetic group of EPO, like most other peroxidases, is protoheme. This conclusion is consistent with other studies in which the optical and EPR properties of EPO derivatives were shown to be very similar to LPO, a protoheme-containing enzyme [16,17]. Hyperfine splitting of the EPR signal from the ferrous nitrosyl derivatives has established that one of the axial ligand atoms is nitrogen for both enzymes, as well as for MPO [32]. In LPO, the axial group has been shown to be a histidine imidazole ring [33]; because the ligand field parameters of other low-spin derivatives of LPO and EPO are very similar, it is likely that histidine is also axially bound to the EPO heme. Thus, at least 5 of the 6 ligation sites may be structurally isomorphous in the 2 enzymes.

The resonance Raman spectra of LPO and EPO, although qualitatively quite similar, compare less favorably than EPR and optical properties. Specifically, the skeletal deformation modes in the high frequency region (ν_{10}, 1613 cm^{-1}; ν_2, 1581 cm^{-1}; ν_{11}, 1557 cm^{-1}; ν_3, 1512 cm^{-1}; ν_{28}, 1477 cm^{-1}) for human EPO are all shifted 5–10 cm^{-1} to lower energies relative to LPO, with the horse enzyme exhibiting intermediate shifts. The positions of these bands have been shown to

correlate inversely with the porphyrin core size in protoporphyrins [30]. Accordingly, the porphyrin ring size is larger in EPO, which implies that the heme iron assumes a more in-plane position in this enzyme. In-plane alignment would be favored if the 6th ligand were more strongly σ-donating in EPO than LPO. Also, the position of the ν_4 band, at 1365 cm^{-1} in EPO and 1373 cm^{-1} in LPO, is considered to monitor the extent of π-delocalization in the porphyrin ring [25,34]. By this criterion, the ring density is greater in native EPO than LPO, again consistent with the presence of a stronger field 6th ligand. Alternatively, this shift could arise from differing protein-heme interactions, e.g. π-overlap with aromatic residues, in the 2 enzymes. Unlike EPO, LPO is unable to catalyze chloride ion peroxidation despite the overall similarity of the heme environments. It may be that subtle structural variations of the type revealed in the Raman spectra can ultimately account for the reactivity differences, e.g., in terms of differing Compound I reduction potentials, but the molecular bases for such effects are not presently understood.

The MPO chlorin is also 6-coordinate, high spin, with axial ligands including nitrogen and a second, relatively weak-field group [5–7,23,32,33]. Since both EPO and MPO are able to use chloride ion in catalyzing halogenation reactions, the MPO chlorin is not obligatory for such activity. It has been noted, however, that the cellular toxicity of EPO-H_2O_2-Cl^- reaction systems is substantially less than MPO-H_2O_2-Cl^- [35]. In these reactions, the selectivity of EPO for iodide and bromide over chloride appears much greater than in the MPO assay systems. The capabilities of EPO towards chloride ion peroxidation are therefore substantially less than MPO, indicating markedly diminished catalytic efficiency for the porphyrin-containing enzyme. It has also been reported that EPO is unable to catalyze certain chlorination reactions that are facile for MPO, e.g. amino acid decarboxylations that proceed via chloramine intermediates [36]. Myeloperoxidase can catalyze formation of free HOCl [1], which is then thought to react chemically as opposed to enzymatically, with substrate. If EPO also serves just to catalyze the formation of HOCl, no fundamental reactivity differences would be expected for the two systems. The catalytic mechanisms may therefore differ; one possibility is that EPO halogenation entails interaction of substrate with an enzyme-hypohalite complex. The observations that LPO [37] and chloroperoxidase [38], enzymes which appear to react by this mechanism, can form freely diffusible hypohalous acids in the absence of substrate, do little to support this contention, however. The question of halide specificity in peroxidase-catalyzed halogenation mechanisms remains an intriguing puzzle.

ACKNOWLEDGEMENT

This research was supported by grants from the National Institutes of Health to J.K.H. (AI-15834) and S.J.K. (AI-07763) for which the authors are deeply grateful.

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