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Photochemically Modified Myeloperoxidase, with Optical Spectral Properties Analogous to Those of Lactoperoxidase, Retains Its Original Catalytic Activity†

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ABSTRACT: During the course of a reducing reaction using ketyl radicals generated from ketone photoreduction with ultraviolet light, a photoinduced chemical modification of the chromophore group in myeloperoxidase has been found. Light absorption and resonance Raman spectra for this modified enzyme indicated an iron porphyrin chromophore group. The alkaline pyridine hemochrome of the modified enzyme exhibited an optical spectrum closely related to that of iron protoporphyrin IX. The chromophore group of the modified myeloperoxidase was cleaved from the protein by methoxide. Proton magnetic resonance of the diamagnetic bis(cyanide) compound of the extracted heme group showed the presence of two vinyl and three methyl side chains associated with a porphyrin macrocycle. These data provide further insight into the structure of the active site in myeloperoxidase. The EPR spectral properties and enzymatic activities of the native myeloperoxidase are essentially conserved in the modified enzyme. Our present results indicate that the heme peripheral substituent is modified while the stereochemical structure surrounding the chromophore group is not altered by the photochemical modification.

Myeloperoxidase is a major component of the antimicrobial system of polymorphonuclear neutrophils (Klebanoff & Clark, 1978). One of the unique properties of myeloperoxidase is its ability to catalyze the formation of hypochlorous acid from hydrogen peroxide and chloride ion (Harrison & Schultz, 1976). Despite many investigations of the spectroscopic and enzymatic properties of myeloperoxidase (Schultz, 1980), the chemical structure of the heme group in this enzyme has not so far been identified because of the inability to extract the heme prosthetic group from the enzyme protein by usual methods, possibly due to the covalent linkage between the heme group and the apoprotein. Nevertheless, several proposals for the chemical structure of the heme group of this enzyme have appeared in the last two decades. Myeloperoxidase, in its reduced form, has a strong absorption band far to the red of α-bands of other reduced heme proteins containing iron protoporphyrin IX. This unusual spectral property is very similar to that of sulfmyoglobin (Nicholls, 1961). It thus seemed that the iron center in myeloperoxidase was a sulfheme or an iron chlorin (Newton et al., 1965a,b). From the reaction of carbonyl reagents with myeloperoxidase, it was suggested that this enzyme might have a formyl substituent in the heme prosthetic group (Harrison & Schultz, 1978; Wu & Schultz, 1975; Odajima, 1980). The spectrum of the alkaline pyridine hemochrome formed from myeloperoxidase was very similar to that of heme *a* obtained from cytochrome oxidase, which contains a formyl group at the 8-position and a vinyl group at the 4-position; thus at least two conjugated electrophilic groups on opposite pyrroles could be considered, implying that the iron complex of 2,4-divinyl-8(5)-formyldeuteroporphyrin IX was the heme of myeloperoxidase (Wu & Schultz, 1975; Harrison & Schultz, 1978). Recent magnetic circular dichroism and resonance Raman scattering studies (Eglinton

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et al., 1982; Sibbett & Hurst, 1984; Babcock et al., 1985; Ikeda-Saito et al., 1985; Sono et al., 1986) have shown that the chromophore group in myeloperoxidase has an effective symmetry lower than that assumed by iron protoporphyrin IX, which led to a proposal that the chromophore group is an iron chlorin and that Raman lines for neither formyl nor vinyl stretching mode were detected. On the other hand, the chemical structure of the heme prosthetic group of lactoperoxidase has been studied by using a reductive cleavage with mercaptoethanol in 8 M urea; it was concluded that the prosthetic group of the enzyme is an iron complex of 2,4-divinyl-8-(mercaptomethyl)deuteroporphyrin IX that forms a disulfide bridge with a cysteine residue (Nichol et al., 1987).

During the course of photochemical reduction of the ferric iron in myeloperoxidase, we have found an unexpected change in the color of the enzyme. Chemical modification of the iron prosthetic chromophore group without disruption of the enzyme protein has been achieved for the first time. In this paper we report our initial characterizations of the novel modified myeloperoxidase by light absorption, EPR,[1] and resonance Raman spectroscopies, as well as its enzymatic activity. The chemical structure of the heme group of the modified enzyme was also deduced from the proton NMR of the diamagnetic ferrous bis(cyano) form of the heme group extracted by the methoxide method of Wu and Schultz (1975). We have reached the conclusion that the unusual optical characterisitics of myeloperoxidase are not a prerequisite for the unique ability of the enzyme to peroxidize chloride ion.

Experimental Procedures

Myeloperoxidase was purified from bovine spleen as described previously (Ikeda-Saito, 1985). The enzyme preparation had an A_{430nm}/A_{280nm} greater than 0.82. A commercial preparation of bovine lactoperoxidase (Sigma) was dissolved in 10 mM phosphate buffer, pH 6.0, applied to a column of CM-Sepharose Cl-6B equilibrated with the same buffer, and eluted with a linear gradient of 10 mM phosphate buffer, pH 6 (starting buffer), and 0.1 M phosphate buffer, pH 7 (limiting buffer). The lactoperoxidase fractions with A_{412nm}/A_{280nm} greater than 0.85 were pooled, concentrated by ultrafilitration (Amicon Centricon 30), and gel-filtered on a column of Sephacryl S-200 equilibrated with 0.1 M phosphate buffer, pH 7. Fractions with A_{412nm}/A_{280nm} larger than 0.9 were pooled and concentrated as above. The preparations used in this study had A_{412nm}/A_{280nm} larger than 0.92.

Photochemical modification was carried out by following the acetophenone–isopropyl alcohol mediated photoreduction method developed by Ward and Chang (1982) for reduction of heme irons from ferric to ferrous states. About a 20-min irradiation of the anaerobic samples (2 mL of 0.1 mM enzyme in 0.1 M phosphate buffer, pH 7) with unfiltered light from a mercury lamp operating at 75 W was sufficient to yield the stable ferrous modified enzyme via the ferrous native enzyme as shown by the optical spectral change (see Figure 1). Upon exposure of the reaction mixture to air, the ferrous modified enzyme was smoothly converted to the brownish ferric modified enzyme.

SDS–PAGE was carried out as described previously (Ikeda-Saito et al., 1989). Enzymatic properties were studied by measuring the rate of hypochlorous acid formation detected by following the chlorination of monochlorodimedone (Sigma), using the technique described by Hager et al. (1966) for

[1] Abbreviations: EPR, electron paramagnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NMR, nuclear magnetic resonance.

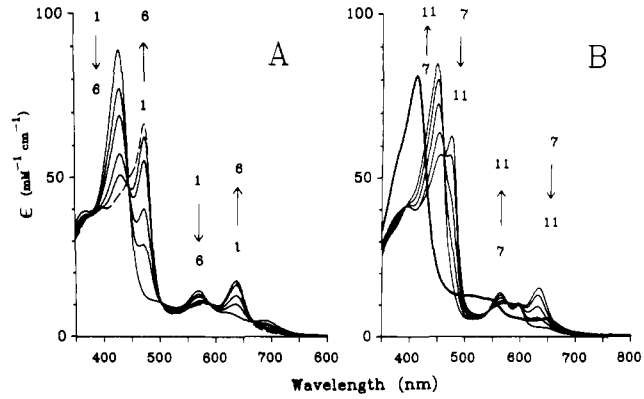

FIGURE 1: The light absorption spectral change associated with the reaction process of ferric native myeloperoxidase to ferrous MPOX via the ferrous native enzyme. Experiments were carried out with 10 μM myeloperoxidase in 0.1 M phosphate buffer containing 0.008% acetophenone and 2% isopropyl alcohol. (A) Photoreduction of ferric to ferrous myeloperoxidase. Total illumination time 0, 120, 210, 390, 570, and 1020 (broken line) s. The arrows indicate the progression of the reaction. (B) The formation of ferrous MPOX from ferrous native myeloperoxidase with successive UV illumination; total illumination 1320, 1620, 1920, 2200, and 2820 s. The arrows indicate the progression of photochemical modification. The ferric MPOX formed by exposing ferrous MPOX to air is shown by a solid bold line.

chloroperoxidase, which has been used for myeloperoxidase (Bakkenist et al., 1980; Ikeda-Saito, 1985). Chlorination of monochlorodimedone was spectroscopically monitored at 290 nm at 20 °C. The experimental conditions were chosen to be close to those used for myeloperoxidase previously (Ikeda-Saito, 1985) so that the results on the photochemically modified enzyme can be compared with those on the native species.

Optical spectroscopies used Shimadzu UV-240 or Hitachi U-3210 spectrophotometers. EPR measurements were carried out with a home-built EPR spectrometer equipped with a Varian X-band cavity operating at 9.35 GHz with a field modulation of 0.5 mT at 100 kHz. An immersion double-Dewar flask was used for measurements at 4.2 K. The microwave frequency was calibrated with a frequency counter (Takeda Riken, Model TR5212), and magnetic field was determined by proton NMR of water protons. Resonance Raman spectra were obtained at the Institute for Molecular Science, Okazaki, Japan, by a JEOL400D Raman spectrometer with 441.6-nm excitation from a He–Cd laser (Kinmon Electronics). Frequencies were calibrated with indene as standard. All the frequencies reported herein were accurate within ±1 cm^{-1}. The temperature of the sample was kept at 25 °C, and local heating was avoided by rotating the cell. Proton NMR spectra were recorded on a JEOL GX-500 instrument at 500 MHz at 30 °C.

Results

Anaerobic irradiation by a high-power mercury lamp in the presence of the acetophenone–isopropyl alcohol mediator (Ward & Chang, 1982) converted resting myeloperoxidase to an unexpected new species with a Soret absorption band at 446 nm. With the use of a lower power mercury lamp it was possible to follow the time-dependent spectral change during the anaerobic illumination. A typical result of such experiments is shown in Figure 1. The spectral change at the initial 570 s corresponds to a change of the valence state of the iron from ferric to ferrous, as the new peaks (638 and 473 nm) in spectrum 5 are identical with those of reduced myeloperoxidase. Upon further irradiation, the Soret absorption band of ferrous myeloperoxidase at 473 nm decreased gradually with a concomitant increase in the absorption band at

Table I: Absorption Maxima (in nm) of MPOX and Lactoperoxidase (LPO) Derivatives

compound	Soret	visible				
MPOX[a]						
reduced		446		563	597	(650 s)
reduced + KCN		435		537	566	
reduced + CO		426		541	574	
ferric	380 s	412	515	545		649
ferric + KCl	380 s	414	515	545		650
ferric + KCN		432		557	595 s	
compound III		427		552	590	
py-hemochrome		422		527	559	
LPO						
reduced (pH 8)[a,b]		445		563	598	
reduced + KCN[a,b]		435		538	566 s	571
ferric[a,d]		413	502	541	592	631
ferric + KCN[a]		432		557	595 s	
compound III[a,d]		428		551	590	
py-hemochrome[e]		422		525	563	

[a] This work. py and s denote pyridine and shoulder, respectively. [b] Read from the spectra in Manthey et al. (1986). [c] Kimura and Yamazaki (1978). [d] Kimura and Yamazaki (1979). [e] Sievers (1979).

446 nm, as shown in Figure 1B. These indicate that the modified enzyme is formed from the ferrous form of myeloperoxidase. An exposure of the reaction mixture (spectrum 11) to air or oxygen yields a brownish compound with a Soret absorption band at 412 nm (bold solid line in Figure 1B). When this compound was irradiated under anaerobic conditions once again or when sodium dithionite was added anaerobically, the ferrous compound with the Soret absorption band at 446 nm was reversibly reproduced. The rate of the photochemically modified myeloperoxidase (MPOX hereafter) formation at pH 9 was about twice that at pH 4, and the rate of reduction of the heme iron from ferric to ferrous states appeared to be the rate-limiting step. We have also examined the photochemical reaction of the enzyme without one or both of the photochemical mediators. Without isopropyl alcohol, ferrous MPOX was still formed via ferrous myeloperoxidase as seen in Figure 1. Without acetophenone (or without both reagents), prolonged irradiation (40 min or longer) attained only partial conversion to ferrous myeloperoxidase, and further irradiation generated quite different and more complicated complexes (spectra not shown). The light absorption spectra of myeloperoxidase and MPOX in 6 M guanidine hydrochloride (buffered by 50 mM Tris, pH 7.8) showed Soret peaks at 429 and 410 nm, respectively. SDS–PAGE of MPOX was identical with that of the native enzyme: two major bands are seen corresponding to a 60-kDa heavy subunit and a 15-kDa light subunit (Ikeda-Saito et al., 1989). Addition of borohydride to MPOX did not cause any detectable optical spectral change.

In the presence of chloride ion, the Soret peak of the ferric MPOX was shifted slightly toward longer wavelength as observed in the native enzyme (spectrum not shown), indicating that MPOX forms a chloride complex that is spectroscopically distinct from that of the native enzyme. The ligand-binding properties of the ferric and ferrous MPOX derivatives were examined, and their absorption maxima are summarized in Table I. In comparing the optical data with those of other known heme proteins, we found an unexpectedly close similarity of the optical properties of MPOX to those of lactoperoxidase, which are also listed in Table I. The major difference is seen in the resting enzyme, as compared in Figure 2, which shows the difference in the shape of the Soret band and in peak positions of the charge transfer bands. We also noticed the different behavior between the reduced forms of MPOX and lactoperoxidase. Reduction of lactoperoxidase

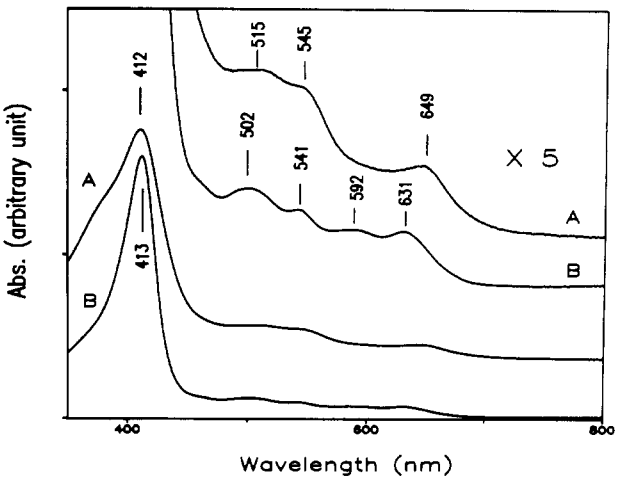

FIGURE 2: Light absorption spectra of ferric MPOX (A) and ferric lactoperoxidase (B) in 0.1 M potassium phosphate buffer, pH 7.0; the spectra of these enzymes are expanded in the visible region.

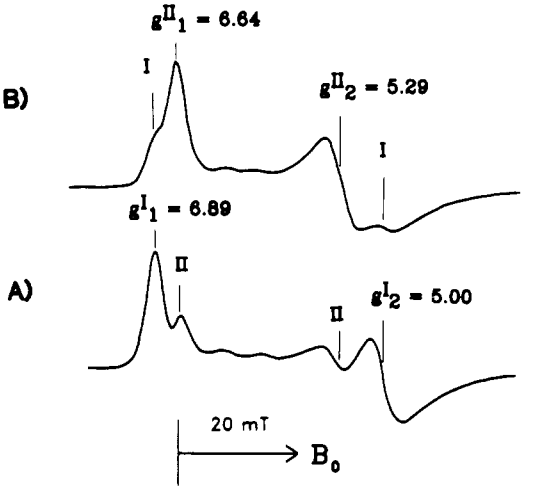

FIGURE 3: X-band powder EPR spectra in the $g = 6$ region of ferric MPOX in 0.1 M phosphate buffer, pH 6, in the absence (A) and presence (B) of 0.2 M chloride.

by sodium dithionite yielded initially an optical spectrum of a "normal" reduced hemoprotein, which after some minutes converted to a more hemochromelike spectrum with an accelerated conversion rate in acidic pH, as reported by Sievers (1980). MPOX did not show any time-dependent change in its optical spectrum upon reduction by sodium dithionite (between pH 6 and 8), and reduced MPOX showed an optical spectrum similar to that of lactoperoxidase immediately after reduction by dithionite at pH 8 (Table I). Although native myeloperoxidase forms stable compound II upon reaction with hydrogen peroxide (Oertling et al., 1988; Ikeda-Saito et al., 1989), spontaneous decay to the resting state of MPOX was observed upon addition of hydrogen peroxide to MPOX. We could not record the optical spectrum of compound II of MPOX by conventional spectrophotometers. The light absorption spectrum of MPOX in the presence of about 100-fold excess of hydrogen peroxide exhibited absorption bands at 590, 552, and 427 (Soret) nm. The same optical spectrum could also be observed as a transient compound during the course of the reaction of ferrous MPOX with air or oxygen to form the ferric compound. We assign this species as compound III of MPOX. The optical spectrum of compound III of lactoperoxidase (Kimura & Yamazaki, 1979) is also very similar to that of compound III of MPOX (Table I).

Figure 3A illustrates a powder X-band EPR spectrum of MPOX (in 0.1 M phosphate buffer, pH 6, without chloride

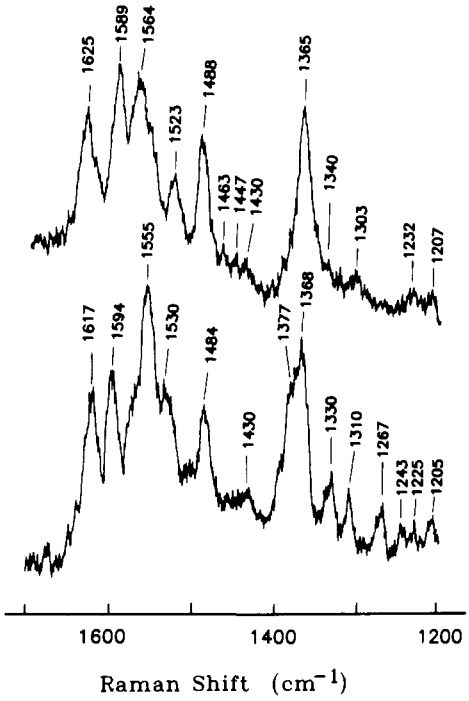

FIGURE 4: Resonance Raman spectra of ferric MPOX (top) and ferric native myeloperoxidase (B) in 0.1 M phosphate buffer, pH 7. Excitation wavelength, 441.6 nm.

ion) in the $g = 6$ region at 4.2 K. This spectrum shows that the modified enzyme is a mixture of two rhombic high-spin species: species I has a larger rhombicity with higher signal amplitude ($g^{I}_{1} = 6.89$ and $g^{I}_{2} = 5.00$) and species II has a smaller rhombicity with reduced signal amplitude ($g^{II}_{1} = 6.64$ and $g^{II}_{2} = 5.29$). Upon lowering the pH to 4.0 (0.1 M citrate–phosphate buffer), the signal amplitude of species I increased while that of species II decreased. In contrast to the changes in the EPR spectrum in the absence of chloride ion, the signal amplitude corresponding to species II increased in the presence of 0.2 M chloride ion at pH 6, as shown in Figure 3B. At pH 4, the signal of species I disappeared upon chloride binding, and species II became the predominant form. At pH 8, signals of both species I and II were observed with almost equivalent signal amplitudes. This indicates that chloride binding induces an increase in rhombicity at high pH and a decrease in rhombicity at low pH. The modified enzyme thus exhibits a change in its high-spin EPR signal upon chloride binding that is rather similar to that seen with the native enzyme (Ikeda-Saito, 1985). As is the case for the native enzyme (Ikeda-Saito et al., 1989), no ferric low-spin signals were observed at higher magnetic fields between pH 4 and 8.

Monochlorodimedone has been used to study the enzymatic activity of myeloperoxidase (Harrison & Schultz, 1976; Bakkenist et al., 1980). Incubation of MPOX with monochlorodimedone, hydrogen peroxide, and chloride resulted in decrease in absorbance at 280 nm, due to the formation of dichlorodimedone (Hager et al., 1966) is observed for the native enzyme. The lineweaver–Burk plot analyses of the chlorination reaction of MPOX at NaCl concentrations between 25 and 200 mM at pH 4.5 show that chloride is not only a substrate of MPOX but also behaves as a competitive inhibitor with respect to hydrogen peroxide, as is the case for the native enzyme (Bakkenist et al., 1980; Ikeda-Saito, 1985). The effects of hydrogen peroxide and chloride concentrations on the chlorination rate for MPOX seem to be similar to those for the native enzyme (Ikeda-Saito, 1985), but the overall

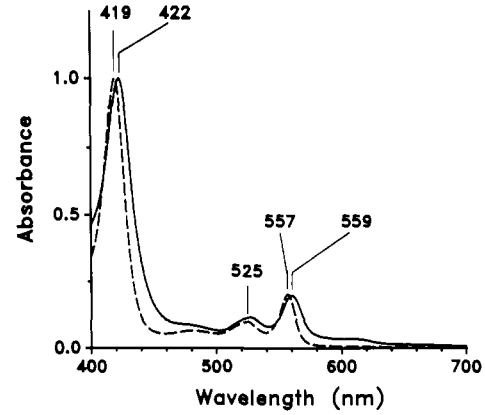

FIGURE 5: Dithionite-reduced alkaline pyridine hemochromogen spectra of MPOX (solid line) and myoglobin (broken line) in 0.1 N NaOH and 50% pyridine.

reaction rate for MPOX is somewhat faster than that of the native enzyme. It is apparent that photochemical modification did not eliminate the original myeloperoxidase enzymatic activity.

Figure 4 compares the high-frequency region of the resonance Raman spectrum of MPOX (top spectrum) with that of native myeloperoxidase (bottom spectrum). Photochemical modification of the enzyme drastically alters the resonance Raman spectral properties; extra lines near the so-called oxidation state marker line (ν_4) of the native enzyme spectrum, which was indicative of the lowered effective symmetry of the chromophore group in native myeloperoxidase (Sibbett & Hurst, 1984; Babcock et al., 1985; Ikeda-Saito et al., 1985), are replaced with a symmetrical singlet ν_4 line (1365 cm^{-1}) in the spectrum of MPOX. The Raman spectrum of MPOX does not resemble that of the a chlorin as does that of the native enzyme.

Figure 5 illustrates the dithionite-reduced alkaline pyridine hemochromogen of MPOX. The spectrum, with absorption bands at 559, 527, and 422 (Soret) nm, is indistinguishable from that of the sodium borohydride modified ferroheme of myeloperoxidase in alkaline pyridine reported by Harrison and Schultz (1978). The spectrum of the pyridine hemochromogen of myoglobin is also shown for comparison. Small shifts toward longer wavelength can be observed in the spectrum of the MPOX complex, suggesting that the substituents of the heme prosthetic group in MPOX are very similar to those of the iron protoporphyrin IX but perhaps with slightly more electrophilic substituents on the heme periphery.

The usual acid butanone method (Teal, 1959) did not extract the heme group from the MPOX protein. Thus, the methoxide method described by Wu and Schultz (1975) for native myeloperoxidase was attempted. After methoxide–methanol treatment, the heme group was extracted into diethyl ether. The extract was dried and washed with water. The dried material was dissolved into 0.1 N NaOD, and undissolved materials were removed by filtration. After an addition of potassium cyanide, the heme was reduced by dithionite under nitrogen in the NMR sample tube to yield the bis(cyano) hemochromogen. In aqueous alkaline solution, the hemochromogen showed absorption bands at 569.5, 539.1, and 436.7 (Soret) nm. Table II lists the chemical shift values of the assignable proton resonances of the ferrous bis(cyano) complex of the extracted heme of MPOX, together with assignment of the peaks based on the NMR spectrum of the bis(cyano) complex of protoheme IX. The major difference between the heme group of MPOX and protoheme IX is that the extracted heme group of MPOX exhibited only *three* sets

Table II: Proton NMR Chemical Shift Values of Ferrous Bis(cyano) Complex of the Extracted Heme Group of MPOX and Protoheme IX[a]

MPOX heme	protoheme IX	assignment
9.51 (1 H, s)	9.49 (1 H, s)	
9.42 (1 H, s)	9.42 (1 H, s)	$-CH-$
9.37 (1 H, s)	9.36 (1 H, s)	meso protons
9.36 (1 H, s)	9.29 (1 H, s)	
8.17 (1 H, m)	8.18 (2 H, m)	$-CH{=}CH_2$
7.87 (1 H, m)		vinyl group
6.22 (2 H, dd)	6.19 (2 H, dd)	$-CH{=}CH_2$
5.93 (2 H, dd)	5.92 (2 H, dd)	vinyl group
4.12 (2 H, t)	4.01 (4 H, t)	$-CH_2CH_2COO^-$
4.04 (2 H, t)		propionate group
3.47 (3 H, s)	3.452 (3 H, s)	
3.43 (3 H, s)	3.449 (3 H, s)	$-CH_3$
3.33 (3 H, s)	3.32 (3 H, s)	methyl group
	3.30 (3 H, s)	
3.08 (2 H, t)	2.99 (4 H, t)	$-CH_2CH_2COO^-$
3.01 (2 H, t)		propionate group

[a] The chemical shift values are in ppm from 2,2-dimethyl-2-silapentane-5-sulfonate. Peak intensities are indicated as 1 H, 2 H, 3 H, and 4 H in the parentheses for intensitites equivalent to one, two, three, and four protons, respectively. The letters s, m, dd, and t in the parentheses represent singlet, multiplet, doublet of doublet, and triplet resonances, respectively. In addition to the peaks listed above, the MPOX heme spectrum exhibited the following unassignable peaks: 5.77 ppm (2 H, s), 3.54 ppm (4 H, m), 2.72 ppm (2 H, s), and two very large peaks at 1.4 and 2 ppm.

of methyl group signals in the spectral region where four sets of methyl group resonances are found for protoheme IX. Another salient feature of the heme of MPOX is that some pairs of protons which are equivalent in protoheme IX are not equivalent in the extracted heme of MPOX. For example, two $-CH{=}CH_2$ protons in protoheme IX resonate at 8.18 ppm with intensity equivalent to two protons, while corresponding resonances are seen at 8.17 and 7.87 ppm as two separate peaks each associated with a single proton intensity. Similar differences are seen for the protons of the methylene groups of two propionic acid side chains $-CH_2CH_2COO^-$. The symmetry of the heme group is likely to be lowered with respect to that of protoheme IX; a possible origin might be a replacement of one of the four methyl group side chains by another group, as reported for the heme group in lactoperoxidase (Nichol et al., 1987) and for the 8-hydroxymethyl derivative of protoheme IX (Ator & Ortiz de Montellano, 1987).

Discussion

Before we discuss the properties of MPOX, the possible modification site should be considered. The SDS–PAGE results shows that the photochemical reaction did not change the subunit structure of the enzyme; i.e., the photoreaction did not cleave the polypeptide chains. Since the modification causes a change in the color of the enzyme, it must occur at the prosthetic chromophore group and/or at the protein moiety very close to the chromophore. If only the protein moiety were modified by the current procedure, the optical spectrum of the heme group free from the protein would be expected to be the same for the native and modified enzyme preparations. The optical spectra of the modified enzyme under denaturing conditions, i.e., the spectrum in 6 M guanidine hydrochloride or in alkaline pyridine, differ from those of the native enzyme under the same denaturing conditions. Thus, the chromophore, the prosthetic group of the enzyme, must be modified. The presence of a single set of isosbestic points between the ferrous native enzyme and ferrous modified enzyme during the course of the photochemical modification argues against additional reactions in the protein near the heme group. Thus, we conclude that the photochemical modification procedure used here for myeloperoxidase only alters the chemical structure of the prosthetic heme group of the enzyme.

Odajima and Yamazaki (1972) reported "acid-treated myeloperoxidase" in which the light absorption spectrum was similar to that of cytochrome oxidase but the peroxidase activity was comparable to that of the native enzyme. However, the chloride peroxidation activity, the physiological function of myeloperoxidase, was not studied. Odajima (1980) also reported several modified myeloperoxidase preparations upon treating the native enzyme with carbonyl reagents including borohydride, hydrazine, and benzylhydrazine, but these modified enzymes showed only a trace of peroxidase activity. On the other hand, our present results clearly demonstrate that the photomodified myeloperoxidase retains its original enzymatic properties toward substrates and inhibitors. MPOX exhibits a change in its ferric high-spin EPR signal upon chloride binding in the same manner as the native enzyme (Ikeda-Saito, 1985), an increase in rhombicity at high pH (species I), and a decrease in rhombicity at low pH (species II), although the transition between these two rhombic species in MPOX seems to have higher pK values than in the native enzyme. In addition, the g-values are slightly different from those for the native enzyme. These slight differences might be caused merely from the different electronic structure of the heme in these preparations. Chloride also affects the light absorption spectrum of MPOX, as seen with the native enzyme. Moreover, the steady-state enzyme kinetics experiments with MPOX showed the dual role of the chloride, as substrate and inhibitor, exactly as seen in the native enzyme. The faster overall rate seen in MPOX can be explained by the spontaneous decay of compound II to the resting state in the absence of halide. Bolscher et al. (1984) showed that some compound I of myeloperoxidase decays to inactive compound II instead of reacting with chloride and that the accumulation of the stable compound II of myeloperoxidase decreases the apparent reaction rate. Since compound II of MPOX spontaneously decays back to the resting state, which can then react with substrate hydrogen peroxide, the apparent overall reaction rate is faster in the MPOX reaction than in native myeloperoxidase. These spectroscopic and enzymatic properties of MPOX strongly argue that the stereochemical structure in the active-site vicinity of the functional groups of the native myeloperoxidase are scarcely altered by the photochemical modification.

Although native myeloperoxidase reacts with carbonyl reagents, such as borohydride, to form modified enzymes (Odajima, 1980), MPOX does not have reactivity toward this reagent. The alkaline pyridine hemochromogen of MPOX is similar to that of the borohydride-modified heme of myeloperoxidase (Harrison & Schultz, 1978). It seems likely that the same substituent(s) of the heme group is(are) modified by the photoreduction employed here and by the borohydride reduction. In order to establish the chemical structure of the chromophore group in native myeloperoxidase, a better understanding of the chemistry of this photoreaction is required. However, we are able to discuss the structure of the chromophore group on the basis of the proton NMR and resonance Raman data to some extent, as below.

The light absorption spectra of MPOX derivatives are similar to those of lactoperoxidase. Our recent nuclear Overhauser effect study on the cyanide complex of MPOX showed essentially the same hyperfine-shifted proton resonances and the

inter proton connectivities between pairs of hyperfine-shifted proton sets as those of the cyanide complex of lactoperoxidase.[2] These suggest a close resemblance in the structure of the prosthetic groups in MPOX and lactoperoxidase. Differences are observed, however, in the light absorption spectra of the ferric high-spin species in the charge transfer bands, indicating a difference in the electronic state of the d-orbital of the iron (Eaton & Hofrichiter, 1981). The difference might be reflected in the different EPR g-anisotropy of the ferric high-spin derivatives. The singlet ν_4 (1365 cm^{-1}) band observed in the resonance Raman spectrum of ferric MPOX indicates that the chromophore of the modified enzyme has an effective molecular symmetry as high as that of iron porphyrin, and it can be concluded that MPOX contains an iron porphyrin chromophore, as reported for lactoperoxidase (Kitagawa et al., 1983). It should be noted that there are deviations in the Raman spectra between MPOX and lactoperoxidase. For example, the ν_4 band differs by 8 cm^{-1} and other high-frequency bands typically by 4 cm^{-1}. Further Raman measurements on MPOX derivatives are required to determine the similarity of the heme macrocycle structure of the prosthetic groups between MPOX and lactoperoxidase.

Proton resonances of the extracted chromophore group of MPOX indicate the presence of two vinyl groups. One may suggest that a formyl group or groups were present at the heme periphery in the native enzyme and that the photochemical reaction employed here would have formed vinyl group(s). This is not the case, since photochemical reduction of 2,4-diformylheme yielded a hematohemelike iron porphyrin instead of converting formyl groups into vinyl groups.[3] It can be concluded that there are two vinyl groups present at the periphery of the chromophore group of myeloperoxidase, in accordance with an early proposal based on the Raman spectra of chlorin derivatives (Anderson et al., 1984). One of the most interesting features of the NMR spectrum of the extracted chromophore group of MPOX is that there are only three methyl groups detected. In accordance with the proposed structure of the heme group in lactoperoxidase, it is highly likely that one of the four methyl groups in protoheme IX would be the site for the possible covalent linkage to the apoprotein moiety of the enzyme. The presence of the very large signal at 1.4 and 2 ppm indicates that the long side chain is still attached to the extracted chromophore heme group of MPOX, indicating that the cleavage of a heme side chain by methoxide–methanol did not occur very close to the porphyrin macrocycle. The symmetry reduction observed as inequivalent resonances of the vinyl and propionate group protons could be due to the substitution of one of the methyl groups with an unusually long side chain. The presence of normal propionate groups in the extracted chromophore of MPOX showed that they are not the linkage sites to the protein moiety in MPOX. Instead, we think that one of the positions where methyl groups are involved in protoheme IX is the linkage site to the apoprotein. In addition to the assignable proton resonances, the extracted heme of MPOX exhibited NMR signals at 5.77, 3.54, and 2.72 ppm (Table II). Since they are not assignable to protons of protoheme IX, these resonances are likely due to protons of the linkage site. In the NMR spectrum of the heme group of lactoperoxidase, the internal methylene protons of the $-CH_2SCH_2CONH^-$ group, which replaces the eighth methyl group of protoheme IX, resonate between the vinyl protons and the internal methylene protons of the propionic acid group (Nichol et al., 1987). A similar chemical shift pattern is seen for the methylene protons of the $-CH_2OH$ group in the 8-hydroxymethyl derivative of protoheme isolated from phenylhydrazine-inactivated horseradish peroxidase (Ator & Ortiz de Montellano, 1987). The resonance at 5.77 ppm of the extracted heme of MPOX could be assignable to internal methylene protons of a heme side chain. This implies that one of the methyl groups of protoheme IX is replaced with a $-CH_2X-$ group as a linkage between the heme group and the MPOX protein. Further characterization of the MPOX heme should identify the chemical structure of this side chain.

In conclusion, we are able to prepare an active modified myeloperoxidase with spectroscopic properties of an iron porphyrin prosthetic chromophore. This is an indication that the unusual optical spectroscopic properties of myeloperoxidase are *not* necessarily related to the unique enzymatic activity of the peroxidation of chloride ion. Instead, the salient functional feature of the enzyme is based on its apoprotein structure around the active site.

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Affinity Labeling of Bovine Liver Glutamate Dehydrogenase with 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Diphosphate and 5′-Triphosphate†

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ABSTRACT: Bovine liver glutamate dehydrogenase reacts with 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate (8-BDB-TA-5′-DP) and 5′-triphosphate (8-BDB-TA-5′-TP) to yield enzyme with about 1 mol of reagent incorporated/mol of enzyme subunit. The modified enzyme is catalytically active but has decreased sensitivity to inhibition by GTP, reduced extent of activation by ADP, and diminished inhibition by high concentrations of NADH. Since modified enzyme, like native glutamate dehydrogenase, reversibly binds more than 1 mol each of ADP and GTP, it is unlikely that 8-BDB-TA-5′-TP reacts directly within either the ADP or GTP regulatory sites. The rate constant for reaction of enzyme exhibits a nonlinear dependence on reagent concentration with K_D = 89 μM for 8-BDB-TA-5′-TP and 240 μM for 8-BDB-TA-5′-DP. The ligands ADP and GTP alone and NADH alone produce only small decreases in the rate constant for the reaction of enzyme with 8-BDB-TA-5′-TP, but the combined addition of 5 mM NADH + 200 μM GTP reduces the reaction rate constant more than 10-fold and the reagent incorporation to about 0.1 mol/mol of enzyme subunit. These results suggest that 8-BDB-TA-5′-TP reacts as a nucleotide affinity label in the region of the GTP-dependent NADH regulatory site of bovine liver glutamate dehydrogenase.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme that is activated by ADP and is inhibited by GTP as well as by high concentrations of NADH, both of which bind to a regulatory coenzyme site distinct from the catalytic site (Colman, 1990). The allosteric inhibitor GTP occupies two sites per subunit of the enzyme in the presence of NADH but only one site in the absence of the reduced coenzyme (Pal & Colman, 1979). The allosteric activator ADP also binds to two sites per subunit (Batra & Colman, 1986a).

Previously, group-specific reagents have been used to chemically modify the amino acids in the regulatory and catalytic sites, but in many cases, high incorporation of the reagents complicated the interpretation of the results and the identification of the critical amino acids (Goldin & Frieden, 1972; Eisenberg et al., 1976). Approaches using purine nucleotide analogues as affinity labels have yielded more specific modifications of amino acids at the regulatory and catalytic sites (Colman, 1983).

This study is part of a systematic effort using the strategy of affinity labeling to locate regions of glutamate dehydrogenase critical for its regulatory behavior. The (fluorosulfonyl)benzoyl nucleosides (Colman, 1983, 1989) have their reactive functional groups at a position structurally equivalent to the pyrophosphate region of ATP, GTP, or the nicotinamide ribose of NADH. The adenine nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine was shown to be incorporated into glutamate dehydrogenase at the NADH inhibitory site to the extent of 3 mol/mol of hexameric enzyme, labeling equal amounts of lysine-420 and tyrosine-190 (Pal et al., 1975; Saradambal et al., 1981; Schmidt & Colman, 1984). The fluorescent analogues, 5′-[*p*-(fluorosulfonyl)-benzoyl]-1,N^6-ethenoadenosine and 5′-[*p*-(fluorosulfonyl)-benzoyl]guanosine have been shown to react covalently at a GTP inhibitory site (Pal & Colman, 1979; Jacobson & Colman, 1982). More recently, another class of affinity labels

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