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Structural Analysis of Myeloperoxidase by Resonance Raman Spectroscopy†

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ABSTRACT: Soret excitation of canine myeloperoxidase (MPO) gives rise to a complex resonance Raman (RR) spectrum characterized by multiple bands in the core size and oxidation state marker regions. Relative intensities of the bands obtained by 406- and 454-nm laser excitation were nearly identical and were temperature independent from 77 to 273 K. Spectra of dithionite-reduced and cyanide-coordinated derivatives are also reported. In the native and dithionite-reduced enzyme, there are no detectable bands between 1620 and 1700 cm^{-1}, indicating that the hemes do not contain formyl substituents in conjugation with the macrocyclic ring. Excitation of the visible absorption band at 568 nm gave rise to only very weakly resonance-enhanced spectra. The RR spectra are interpreted within the context of other physical measurements to indicate that MPO contains two equivalent or nearly equivalent chlorin prosthetic groups. Possible mechanistic consequences of these structural features are discussed.

Myeloperoxidase (MPO)[1] has been implicated in the antimicrobial and cytotoxic reactions of neutrophils and monocytes (Klebanoff & Clark, 1978). This enzyme is functionally unique in its capacity to catalyze the peroxidation of chloride ion (Harrison & Schultz, 1976). The reaction produces hypochlorous acid (HOCl), whose ability to cause extremely rapid oxidative degradation of a wide variety of biological substrates, including porphyrins, hemes, and heme proteins (Albrich et al., 1981), suggests that it is the ultimate MPO-generated toxin (Klebanoff & Clark, 1978; Albrich et al., 1981).

The enzyme is dimeric (Andrews & Krinsky, 1981; Harrison et al., 1977; Olsson et al., 1972), containing two heme prosthetic groups (Agner, 1958) which bind small anions (Eglinton et al., 1982; Wever & Bakkenist, 1980; Harrison, 1979; Odajima & Yamazaki, 1972a) and react with hydrogen peroxide (Harrison et al., 1980). During turnover, MPO undergoes slow self-inactivation that is accompanied by chromophoric bleaching (Matheson et al., 1981), but this reaction is inhibited by addition of HOCl-reactive reagents (Naskalski, 1977). Apparently, therefore, the MPO active site is unusually constructed to provide self-protection during formation of the oxidant. The ability of the enzyme to generate large quantities of HOCl in the presence of oxidizable substrates and then inactivate when substrate is depleted may be critical to its proper functioning in the physiological environment. One can envision that phagosomal stimulation by invading pathogens leads to oxidative degradation primarily of internalized organisms, beyond which point HOCl production must be terminated to minimize damage to surrounding host tissues.

Attempts to identify the iron-containing chromophores have been frustrated by their chemical instability (Harrison & Schultz, 1978; Wu & Schultz, 1975; Nichol et al., 1969; Odajima & Yamazaki, 1972b; Newton et al., 1965a,b). The prosthetic groups are covalently linked to the protein (Wu & Schultz, 1975; Schultz et al., 1983). Under conditions examined thus far, cleavage has led to isolation of hemes with physical properties that are distinctly altered from the native enzyme (Harrison & Schultz, 1978; Wu & Schultz, 1975; Nichol et al., 1969). Structural identification has therefore relied principally upon inference from physical characteristics of the intact enzyme. Soret and visible absorption bands, especially of the reduced form, are dramatically red shifted compared to those of protoheme (Adar, 1978) and heme *c* containing peroxidases (Rönnberg et al., 1981). On the basis

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[1] Abbreviations: MPO, myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7); RR, resonance Raman; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism.

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of spectral analogy of the reduced enzyme with ferrous sulfmyoglobin and similar ferrochlorins (Newton et al., 1965a,b; Morell et al., 1968; Stolzenberg et al., 1981), it has been suggested that the prosthetic groups are chlorins (Newton et al., 1965a,b; Morell et al., 1968). Recent support for this notion comes from favorable comparison of the MCD spectrum of MPO with *Pseudomonas aeruginosa* nitrite reductase (Eglinton et al., 1982), which is thought to be a chlorin (heme d_1)-containing enzyme (Walsh et al., 1980). Alternatively, because the pyridine hemochrome spectrum resembles that of heme *a* (Harrison & Schultz, 1978; Wu & Schultz, 1975; Newton et al., 1965a,b), the prosthetic groups have been assigned as formylporphyrins (Harrison & Schultz, 1978; Wu & Schultz, 1975). This suggestion is supported by observations that reaction of the labilized heme with reagents capable of reducing formyl substituents generally forms products with blue-shifted optical spectra, as anticipated for loss of an electrophilic substituent (Harrison & Schultz, 1978; Wu & Schultz, 1976; Odajima, 1980).

Evidence relating to heme equivalence has been reported but is conflicting. Binding inequivalence has been suggested from various extraction procedures which labilize 50% of the heme (Agner, 1958; Harrison & Schultz, 1978), although the recent separation of enzyme into apparently identical, active monomeric subunits (Andrews & Krinsky, 1981; Andrews et al., 1984) suggests that there are no inherent differences in heme–subunit interactions. Electronic spectra, redox titrations (Harrison & Schultz, 1978), and halide binding studies (Bakkenist et al., 1980) give no evidence of differential behavior, although in the latter studies reaction stoichiometries have not been directly determined. Cyanide binding is reported to occur with stoichiometries of 1/1 or 2/1 CN^-/enzyme, i.e., binding either to a single heme (Odajima, 1980) or potentially to both hemes (Eglinton et al., 1982). Cyanide ion binds to both hemes in ferromyeloperoxidase, although coordination is apparently accompanied by conformational distortion of the protein (Harrison, 1979). Reductive titration with dithionite is reported to require only one reducing equivalent per enzyme (Odajima, 1980), yet integration of EPR signals suggests that the resting enzyme contains both iron atoms in the ferric oxidation state (Eglinton et al., 1982). Oxidation of thiocyanate ion by MPO compound II follows biphasic kinetics, each phase contributing approximately equally to the overall reaction (Harrison & Schultz, 1978). Two distinct anion binding sites, catalytic and inhibitory, are recognized in halide (Bakkenist et al., 1980; Andrews & Krinsky, 1982; Harrison, 1976) and pseudohalide (Wever et al., 1982) peroxidation by the enzyme, although only the catalytic site is spectroscopically detectable, suggesting that inhibition may not involve heme ligation. In any event, the data presently do not permit discrimination between heme equivalence or pronounced inequivalence.

Because the MPO hemes are apparently structurally unique and because their reactivity bears significantly upon MPO function in disinfection (Klebanoff & Clark, 1983) and the inflammatory response (Weiss et al., 1983; Henderson & Klebanoff, 1983), we have sought their additional characterization by resonance Raman (RR) spectroscopy. This technique, which has not been applied previously to MPO, has proven useful in identifying heme oxidation and electronic spin states, as well as certain peripheral substituents, e.g., formyl groups (Spiro, 1983).

Materials and Methods

Canine myeloperoxidase was generously provided by Seymour Klebanoff (University of Washington, Seattle, WA). For the native enzyme, a value of 0.72 was measured for the absorbance ratio A_{430}/A_{280}. Typical values reported for highly purified enzyme are 0.7–0.8 (Andrews & Krinsky, 1981; Matheson et al., 1981; Schultz & Shmukler, 1964). Samples were calculated to be 1×10^{-5} M by using $E_{428/mol} = 1.78 \times 10^5$ M^{-1} cm^{-1} (Olsen & Little, 1983). Absorption maxima were observed at 359, 428, 568, 626, and 684 nm. The reduced form of the enzyme was obtained by titration with solid, free-flowing sodium dithionite (BDH Chemicals Ltd., Poole, England) under an argon atmosphere. The progress of the reduction reaction was monitored spectrophotometrically until incremental addition of dithionite caused no further spectral change. Absorption maxima for the reduced enzyme were observed at 468 and 634 nm. The cyanide derivative of MPO was produced via titration with solid potassium cyanide (reagent grade, Baker, Phillipsburg, NJ) following the same procedure as the reductive titration. Absorption maxima were observed at 456 and 632 nm. All absorption spectra taken for MPO and its derivatives were in agreement with those of previous reports (Andrews & Krinsky, 1981; Eglinton et al., 1982; Wever & Bakkenist, 1980; Odajima & Yamazaki, 1972a; Harrison et al., 1980; Matheson et al., 1981; Agner, 1941). The perchlorate salt of tris(2,2′-bipyridyl)cobalt(III), $E_{307} = 3 \times 10^4$ M^{-1} cm^{-1} (Ciana & Crescenzi, 1978), was prepared according to published methods (Berkoff et al., 1980).

Sample excitation was provided by Spectra Physics ion lasers (164-05 argon, 164-01 krypton). The Raman spectrometer and computer interface have been described elsewhere (Loehr et al., 1979); the scattered light was collected by using a 90-deg geometry. Depolarization experiments were conducted with a polarizer situated between the laser head and sample. For most experiments, a stream of cold dry N_2 was used to control temperature at the sample such that freezing at the point of laser incidence was just prevented by internal heating due to laser light absorption. For low-temperature studies, samples were mounted in a copper-rod cold finger immersed in liquid N_2 (Sjöberg et al., 1982). For all Raman experiments, samples were contained in glass capillary tubes. Electronic spectra were obtained directly from these tubes with a Cary 16 spectrophotometer by placing them in the 2–5 °C water-filled chamber of a self-masked 1-cm cuvette. The base lines for these spectra were suitably flat between 400 and 800 nm but subject to large background shifts, thus limiting the utility of the method to qualitative assessments. For quantitative measurements, conventional spectroscopic methods were employed. No detectable photodecomposition of MPO hemes exposed to the laser beam was observed; electronic spectra were identical before and after each experiment, and the Raman spectra remained constant over the 30–300-min periods required for data collection.

Results and Discussion

Carbonyl Stretching Region. Figures 1 and 2 show the RR spectra of native, dithionite-reduced, and cyanide-complexed canine MPO under 454.5-nm excitation. A conspicuous feature of the RR spectra of native and reduced MPO is the absence of bands above 1610 cm^{-1}. A formyl group on the periphery of heme typically gives rise to a RR band in the region 1670–1640 cm^{-1} (Callahan & Babcock, 1983; Van Steelandt-Frentrup et al., 1981; Tsubaki et al., 1980). This band has been assigned to the formyl carbonyl stretching mode and is found over a 30-cm^{-1} range because variations occur in the strength of hydrogen bonding to the carbonyl oxygen (Choi et al., 1983). With Soret excitation, the mode appears to be Raman active for all formyl hemes and heme proteins. A 1610-cm^{-1} band in ferrocytochrome *a* has recently been

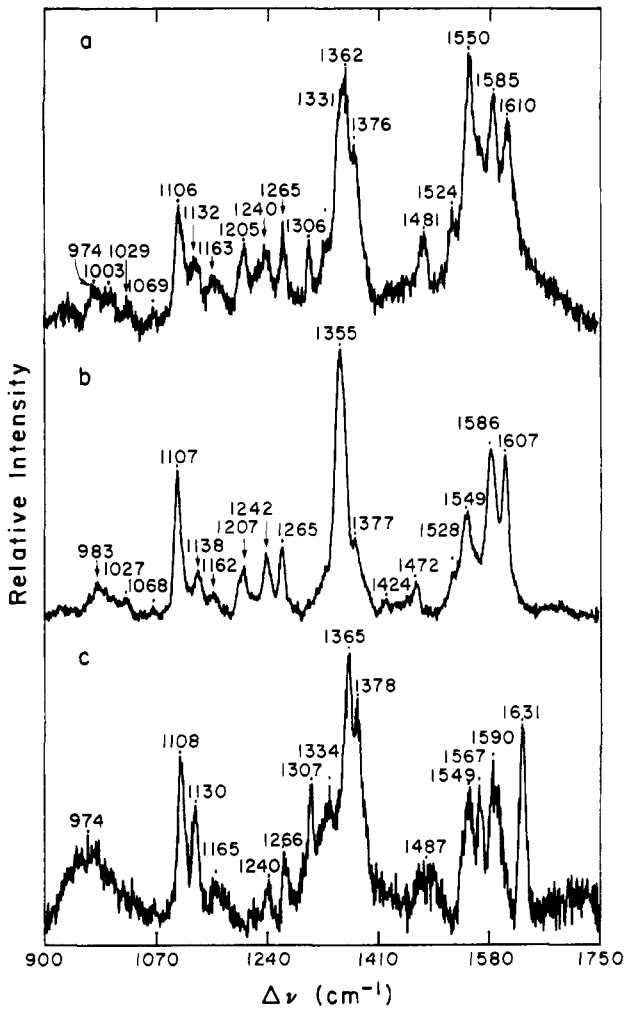

FIGURE 1: Resonance Raman spectra of canine myeloperoxidase, 900–1750 cm^{-1}. (a) Native enzyme; (b) dithionite reduced; (c) enzyme–cyanide complex. The spectra were obtained with 38–40 mW of 454.5-nm excitation and are accumulations of 8–15 scans; scan rate 1.0 cm^{-1} s^{-1}; slit width ~8 cm^{-1}. Spectrum a band frequency and depolarization ratios (ρ): 1585 (0.42); 1550 (0.46); 1362 (0.45); 1265 (0.46); 1242 (0.39); 1205 (0.35); 1161 (0.74); 1107 (0.57); ν_1 SO_4^{2-} 982 (0.06).

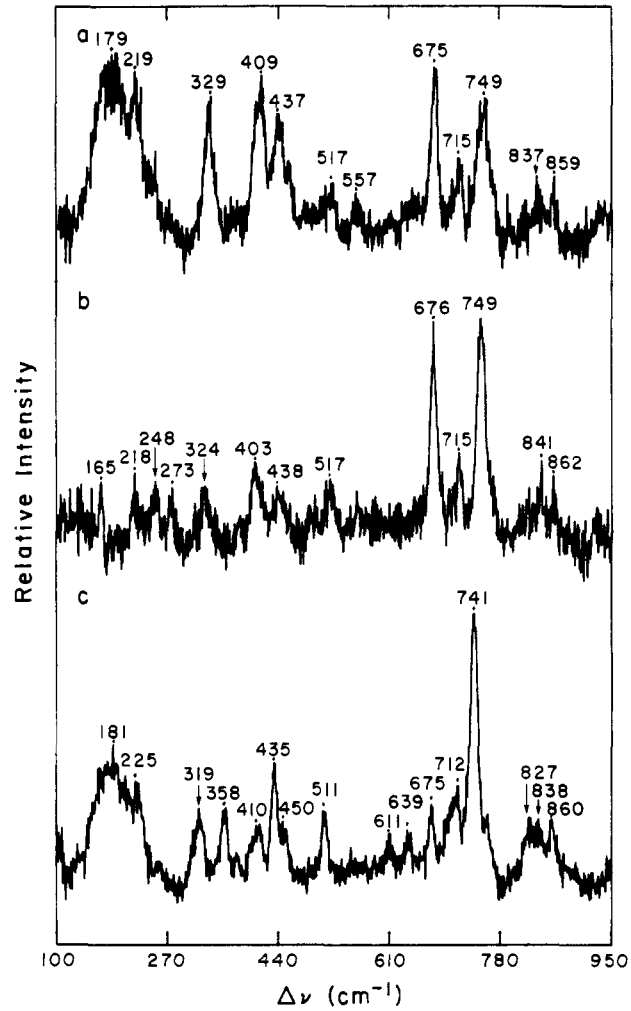

FIGURE 2: Resonance Raman spectra of canine myeloperoxidase, 100–950 cm^{-1}. Conditions and labels as in Figure 1.

assigned to a carbonyl stretching frequency (Callahan & Babcock, 1983); its low frequency is suggested to arise from unusually strong hydrogen bonding between the carbonyl group and protein side-chain substituents. The 1610-cm^{-1} band in MPO does not appear to have a similar origin, however. Unlike heme *a* in cytochrome oxidase, this band in MPO is independent of the iron oxidation state (Figure 1), a finding that is inconsistent with the known resonance interaction between central metal and formyl porphyrin ring substituents (Chang, 1979). The absence of a formyl band indicates that formyl substituents are either not present or not in conjugation with the macrocyclic ring, i.e., out of plane or attached to the pyrroline ring. In all events, the electronic spectrum will be largely unperturbed. Consequently, the most straightforward explanation for MPO's red-shifted electronic spectrum is that its hemes are chlorins (Stolzenberg et al., 1981) or pseudochlorins, as previously proposed (Morell et al., 1968; Nicholls, 1961).

The band at 1610 cm^{-1} is probably the analogue of the ν_{10} mode of Ni(octaethylporphyrin) (Abe et al., 1978). On the basis of work in which the ν_{10} frequency was correlated with the nature of the axial ligand and the coordination number of iron(III) octaethylporphyrinato complexes (Teraoka & Kitagawa, 1980), we attribute the relatively low frequency of the 1610-cm^{-1} band to an exceptionally weak axial field felt by the heme iron. Such weak field ligands might be H_2O, chloride ion, or carboxylate ion. Lactoperoxidase (Sievers et al., 1983) and *Ps. aeruginosa* cytochrome *c* peroxidase (Rönnberg et al., 1980) are precedents for carboxylate as an axial ligand.

Core Size and Oxidation State Marker Regions. Two RR bands appear in each of the core size and oxidation state marker regions, at 1550/1588 and 1362/1376 cm^{-1}, respectively (Figure 1a), whereas typically single hemes give only one band in those regions (Spiro, 1983). The relative intensities of these bands are unchanged upon freezing to 77 K, both with 454- and 406-nm laser excitation (Figure 3), suggesting that the hemes are not present as equilibrium distributions of differing spin states. These general spectral features would therefore appear to dictate either (i) that the hemes are hydroporphyrins, for which ring reduction causes symmetry lowering, thereby increasing the number of totally symmetric, hence Raman-allowed, vibrational modes (Ozaki et al., 1979), or (ii) that the spectrum of the native enzyme consists of overlapping contributions from two vibrationally distinct hemes.

Changes in relative peak intensities upon dithionite reduction are qualitatively consistent with the latter interpretation. The band at 1376 cm^{-1} is substantially reduced in intensity, whereas the 1362-cm^{-1} band increases in intensity relative to, e.g., peaks at 1607 and 1107 cm^{-1}; similarly, the band at 1588 cm^{-1} increases in intensity at the expense of the 1550-cm^{-1} band (Figure 1b). Thus, titration with dithionite suggests the si-

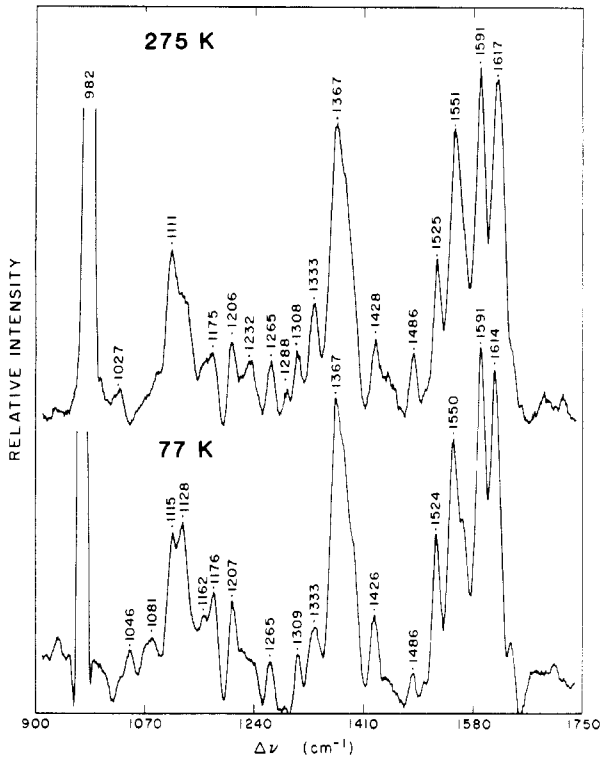

FIGURE 3: Resonance Raman spectra of native canine myeloperoxidase. Upper, 275 K; lower, 77 K. The spectra were obtained with 406.7-nm excitation; scan rate 1.0 cm^{-1} s^{-1}; slit width ~8 cm^{-1}. The upper spectrum is the accumulation of 3 scans with 28–30 mW; the lower spectrum is the accumulation of 10 scans with 33–35 mW. After background subtraction, both spectra were subject to a 25-point smooth.

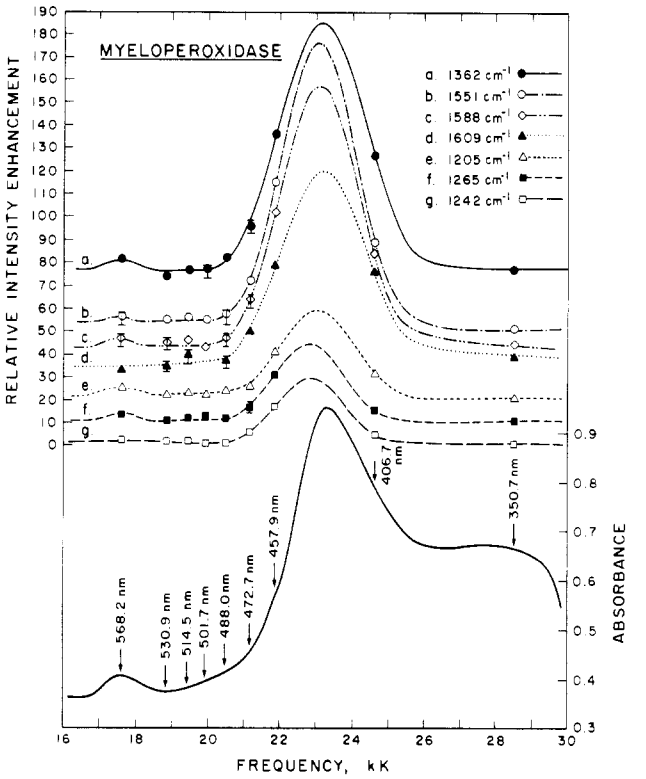

FIGURE 4: Relative intensity enhancement profiles for native canine myeloperoxidase. Intensities were measured relative to the 982-cm^{-1} band of $(NH_4)_2SO_4$ (~0.5 M). The only error bars shown are those in the region below 21.2 × 10^3 K which exceeded the height of the symbol. Curves a–f between 20 × 10^3 and 26 × 10^3 K are shown as the Gaussian fit to the data. For clarity, each profile is displaced 10 units above the next lower profile (except curve a, which is displaced 20 units). The MPO absorption spectrum, given at the bottom of the figure, is marked by arrows indicating laser excitation frequencies.

multaneous presence of high-spin ferriheme and low-spin ferroheme in the enzyme, with reduction converting high-spin ferriheme to the low-spin ferro form. Given this interpretation, the spectral shift from 1610 to 1631 cm^{-1} observed upon titration of native MPO (Figure 1c) with potassium cyanide might be attributed to high-to-low spin conversion of the ferriheme. For six-coordinate ferrihemes, the band assigned to the ν_{10} vibrational mode characteristically appears at 1615–1625 cm^{-1} for the high-spin state (Teraoka & Kitagawa, 1980) and at 1638–1641 cm^{-1} for the low-spin state (Rakshit & Spiro, 1974). The RR spectra of horseradish peroxidase (Teraoka & Kitagawa, 1981), intestinal peroxidase (Kimura et al., 1981), lactoperoxidase (Kitagawa et al., 1983), and cytochrome *c* (Teraoka & Kitagawa, 1980) are consistent with these empirical rules. Cyanide titration of MPO has been shown by EPR, MCD (Eglinton et al., 1982), and magnetic susceptibility studies (Dunford & Stillman, 1976; Ehrenberg, 1962) to increase the content of low-spin heme.

Quantitatively, however, this analysis appears unsatisfactory. High-to-low spin conversion should cause loss of the high-spin marker band at 1549 cm^{-1}, but there is, at best, only slight loss of intensity[2] (Figure 1a,c). Similarly, the apparent inability to cause complete reduction of the ferriheme by dithionite addition is difficult to understand,[3] as is the apparent ferroheme band shift from 1362 to 1355 cm^{-1} (Figure 1a,b). Finally, the oxidation state marker band is insensitive to titration with $Co(bpy)_3^{3+}$ ion (data not shown). This reagent is sufficiently strongly oxidizing (E^0 = +310 mV; Ciana & Crescenzi, 1978) to convert any ferrous heme present in MPO to the ferric state ($E^{0\prime}$ = +36 mV; Harrison & Schultz, 1978). Minimally, a corresponding hypsochromic shift in the composite oxidation state band would be anticipated, arising from a proportionately greater contribution by the higher frequency (1376 cm^{-1}) ferriheme band.

Although the RR of hydroporphyrins is not well-documented, the spectra from several high- and low-spin ferrichlorins are quite similar to those of their complementary porphyrins with the exception that extra bands indeed appear throughout the high-frequency marker regions. This general behavior has been seen for ferrioctaethylchlorins (Ozaki et al., 1979), ferriphotoprotoporphyrin, and ferrideuterochlorin (L. A. Andersson, T. M. Loehr, C. K. Chang, and A. G. Mauk, unpublished results), as well as metaquosulfmyoglobin (L. A. Andersson, personal communication), a pseudochlorin (Morell et al., 1968). Furthermore, apart from the appearance of additional bands, the vibrational assignments given for metalloporphyrins (Choi et al., 1983) appear to be valid irrespective of the extent of ring reduction (Ozaki et al., 1979). The Soret-excited RR spectra of MPO are therefore fully consistent with assignment of the MPO hemes as chlorins.

Enhancement Profiles. The excitation profile for MPO is given in Figure 4. For all Raman bands, including those not shown in Figure 4, only negligible resonance enhancement is observed with laser excitation into the enzyme's visible absorption band at 568 nm. This contrasts with visible excitation of porphyrin-containing proteins; oxy- and methemoglobins for example show a 6–17-fold increase in scattering intensity

[2] Previous EPR and MCD studies have found that a fraction of the heme is resistant to cyanide-induced spin-state changes (Eglinton et al., 1982).

[3] Myeloperoxidase preparations contain several isozymes exhibiting differing kinetic behavior (Feldberg & Schultz, 1972; Pember et al., 1982). This microheterogeneity may contribute as well to the complexity seen in the physical characterization studies.

as resonance is approached in this region (Strekas & Spiro, 1973; Strekas et al., 1973). With long sampling times and maximally concentrated samples, the signal-to-noise ratio of our best 568-nm excitation Raman spectra were on the order of unity. Consequently, we were unable to quantify depolarization ratios. Qualitatively, we observed no inversely polarized bands; within the rough limits of the signal-to-noise ratio, most, if not all, bands appeared polarized.

A possible explanation for these results follows a previous interpretation of the weakly resonance-enhanced Raman spectrum of horseradish peroxidase compound I (Felton et al., 1976). According to theoretical calculations (Weiss, 1978), (1) the lower molecular symmetry of a dihydroporphyrin lifts the degeneracy of the lowest unoccupied and highest occupied orbitals of the four-orbital model, (2) the resulting excited states are nonidentical and do not undergo the sort of configuration interaction which leads, in the case of the porphyrins, to an intense Soret band and weak visible bands, and (3) the symmetry of the dihydroporphyrin molecular states thus permits transitions in the visible region which are directly allowed, and not vibronically mixed. If chlorin visible bands contain little or no vibronically allowed component (Felton et al., 1976), then B-term activity will be diminished and the chlorin Raman spectrum will consist primarily of A-term modes.[4] The intensity dependence of these modes on the allowedness of the resonant electronic transition might therefore limit resonance enhancement of chlorin Raman bands to excitation into a fairly intense absorption band such as the Soret. For some chlorins, this condition conceivably could also be met by excitation into bands in the visible region. For example, the 568-nm excitation near the prominent visible absorption bands of Fe(octaethylchlorin)Cl yields well-resolved RR spectra that consist primarily of polarized A-term modes.[5] On the other hand, the 568-nm absorption band of MPO might be insufficiently intense for appreciable resonance enhancement to occur by an A-term scattering mechanism.

Heme Equivalence. Ps. aeruginosa cytochrome *c* peroxidase contains two structurally and functionally distinct *c*-type hemes. Physical characterization by a variety of techniques has shown that the resting enzyme contains one low-spin and one high-spin ferriheme (Rönnberg et al., 1980; Aasa et al., 1981). Kinetic analysis suggests that the catalytically active form of the peroxidase is a 1-electron-reduced ferro–ferriheme; the high-spin ferriheme is thought to be the site of peroxidative action, while the low-spin ferroheme serves an electron-transport function (Rönnberg et al., 1981). Since MPO is dimeric, a similar bifunctional organization is possible.[6] If the hemes have distinct electronic spectra, then they might be distinguished by selective enhancement of one or the other sets of RR bands as the excitation wavelength is varied across the Soret envelope. If the electronic spectra are coincident, then the relative RR band intensities will be wavelength independent. The RR spectra of native MPO obtained with 454- and 406-nm excitation differ in some respects. Most notably, the asymmetric doublet peak (1362/1376 cm^{-1}) in the oxidation state marker region under 454-nm excitation (Figure 1) is seen as a broad asymmetric band centered at 1367 cm^{-1} under 406-nm excitation (Figure 3). Also, the intensity of the 1550-cm^{-1} band is diminished slightly in the 406-nm spectrum relative to the other peaks in the core size marker region. Although this wavelength dependence might arise from inequivalent hemes, effects of similar character are apparent in the published spectra of Fe(octaethylchlorin)Cl which cannot be attributed to selective enhancement (Ozaki et al., 1979). Our data, therefore, provide no compelling evidence for heme inequivalence.

Functional Roles for Chlorin in Chloride Peroxidation. Ring oxidation, e.g., in forming compound I, the intermediate reactive toward chloride ion (Harrison, 1976), is probably thermodynamically favored for chlorins relative to porphyrins (Stolzenberg et al., 1981), so that the subsequent chloride ion oxidation is energetically disfavored. Therefore, a unique role for active-site chlorins in MPO catalysis probably cannot be rationalized on thermodynamic grounds. In general, rates of oxidation or chlorination by HOCl are extraordinarily sensitive to the nucleophilic character of the site of attack on the reactant partner, indicating that electron transfer requires incipient bond formation (Held et al., 1978; Hurst et al., 1981). The methine bridge positions adjacent to the pyrroline ring in chlorins are relatively electron rich. Consequently, chlorination occurs rapidly at these sites under conditions where analogous porphyrins are unreactive (Woodward & Skaric, 1961; Bonnett et al., 1966). The predominant reaction between chloride ion and metalloporphyrin π-cation radicals appears to be electron transfer, rather than chlorination (Smith et al., 1979). Chloride ion oxidation most likely occurs through a transition state with similar properties, however, so that reactivity will be controlled by the electrophilic character of the oxidant electron-acceptor site. In ferryl π-cations derived from chlorins the electronic ground state is calculated to have a_2 symmetry, for which certain methine positions are relatively electron deficient (Hanson et al., 1981) and may therefore uniquely serve as peripheral ring sites for chloride oxidation by MPO compound I. Assuming that rapid intramolecular electron transfer between the chlorin ring and ferryl iron is possible (Johnson et al., 1978), direct formation of HOCl by a functionally two-equivalent oxidation of chloride ion could occur at this site.

[4] The Raman scattering from a porphyrin has two basic sources (Albrecht & Hutley, 1971). The first source, A-term activity (Franck–Condon scattering), is indicated by the enhancement of the porphyrin's totally symmetric modes. The intensities of these A-term modes depend on the magnitude of the transition dipole moment of the resonant electronic state, and inversely upon bandwidth. Hence, large enhancement is observed with excitation into strongly allowed electronic bands. With Soret excitation, A-term scattering dominates a porphyrin Raman spectrum. The second source of Raman scattering is B-term activity (Herzberg–Teller scattering), characterized by resonance enhancement of vibrations involved in vibronic mixing of the Q and B electronic states. Theory predicts for a porphyrin macrocycle of D_{4h} symmetry that these modes are depolarized or anomalously polarized (Spiro & Strekas, 1972). The intensities of these modes depend on the product of the transition dipole moments of the mixing states (i.e., the magnitude of the mixing integral). For some porphyrins, the visible excitation Raman spectrum is dominated by B-term scattering, whereas for other porphyrins the A- and B-term contributions are comparable.

[5] Inspection of Figures 3 and 4 in Ozaki et al. (1979) reveals that polarized bands increasingly predominate as the excitation wavelength approaches the absorption maximum near 600 nm.

[6] For example, an electron transport heme might play a regulatory role in MPO catalysis. Compound I is the active intermediate for chloride peroxidation; compound II is unreactive toward chloride ion (Harrison, 1976). During turnover, compound II can apparently be reactivated for chloride peroxidation by reduction with H_2O_2 in a step that forms part of an overall catalatic cycle (Harrison, 1982). Because the enzymatic rate of formation of HOCl depends upon the amount of MPO present as compound I, it is a sensitive function of the steady-state concentration levels of H_2O_2. Rapid electron transfer from a regulatory heme site, inactivating the catalytic site, would provide the means for immediate response to changing oxidant levels. During peak H_2O_2 production accompanying maximal phagocytic stimulation, HOCl would form at an optimal rate, but as H_2O_2 levels decline toward the end of the phagocytic respiratory burst, MPO would be proportionately inactivated. In this way, the extent of the enzymatic response could be linked to the magnitude of the stimulating signal.

Alternatively, it has been noted that the greater flexibility of hydroporphyrin rings brought about by disruption of π conjugation permits relatively easy adjustments in core size, facilitating central metal binding of diverse ligands (Strauss et al., 1983). The binding affinity of the weak-field chloride ion may likewise be promoted in the MPO chlorin.

Acknowledgments

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Registry No. Peroxidase, 9003-99-0.

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Red-Edge Excitation of Fluorescence and Dynamic Properties of Proteins and Membranes†

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ABSTRACT: In moderately polar and viscous solvents, the emission spectra of fluorophores often shift to longer wavelengths as the excitation wavelength is increased toward the long-wavelength (red) side of the absorption. Red shifts occur because long-wavelength excitation results in photoselection of those fluorophores which are interacting most strongly with the polar solvent molecules. The observation of excitation red shifts requires that these enhanced dipole–dipole interactions are maintained in the photoselected population during the lifetime of the excited state. Consequently, the magnitude of the excitation red shifts depends upon the dynamic properties of the environment surrounding the fluorophore, as well as upon the solvent polarity and the sensitivity of the fluorophore to the polarity of the solvent. We used this phenomenon to investigate the dynamic properties of reference solvents, model membranes, and the protein apomyoglobin labeled with 6-(*p*-toluidinyl)-2-naphthalenesulfonic acid (TNS). The spectral shifts and lifetime data indicate that red-edge excitation results in the selective excitation of "solvent-relaxed" fluorophores. By comparison of the data obtained for TNS in solvents and bound to the macromolecules, one may estimate the relaxation rate of the environment. This comparison indicates rapid spectral relaxation of TNS bound to lipid vesicles and a somewhat slower relaxation around TNS bound to the heme site of apomyoglobin.

During the past 10 years, fluorescence spectroscopic methods have been widely utilized to investigate the dynamic properties of proteins and membranes. A variety of fluorescence methods have been employed, such as steady-state, time-resolved, and phase-shift methods. Underlying these diverse approaches has been the use of basically two phenomena. These are the permeability of the macromolecule to quenchers [see Calhoun (1983a,b) and references cited therein; Lakowicz & Weber, 1973a,b] and the rotational diffusion of proteins or membrane-bound fluorescent probes [see Lakowicz (1980, 1983) and references cited therein]. Collisional quenching of fluorescence can reveal the dynamics of biopolymers because contact between the fluorophore and quencher is required for quenching. The quenching of a fluorophore buried in a macromolecule requires diffusion of the quencher through the closely packed macromolecule. Similarly, the rotational motion of a fluorophore located internally in a macromolecule is determined by the structural fluctuations of the residues which surround the fluorophore. The phenomenon of time-dependent spectral relaxation has also been utilized, but to a somewhat more limited extent, to quantify the dynamics of macromolecules (Brand & Gholke, 1971; Easter et al., 1978; Lakowicz & Hogen, 1981).

In this paper, we describe a less widely used and understood fluorescence phenomenon and its application to estimating the dynamic properties of proteins and membranes. This phenomenon is the dependence of the fluorescence emission spectra and lifetimes upon the excitation wavelength. This dependence upon excitation wavelength is maximal at low temperatures in polar viscous solvents. The dependence of the emission

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