Photodissociable Endogenous Ligand in Alkaline-Reduced Cytochrome *c* Peroxidase Implicates Distal Protein Tension†

Giulietta Smulevich,*,‡ Mark A. Miller,§ David Gosztola,∥ and Thomas G. Spiro*,∥

Dipartimento di Chimica, Università di Firenze, Via G. Capponi 9, 50121 Firenze, Italy, Department of Chemistry, University of California at San Diego, La Jolla, California 92093, and Department of Chemistry, Princeton University, Princeton, New Jersey 08544-1009

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ABSTRACT: Laser excitation of alkaline- (pH 8.5) reduced cytochrome *c* peroxidase (CCP) produces resonance Raman (RR) bands arising from both low- and high-spin heme species (ν_3 = 1493/1471 cm^{-1}) even though in the absence of laser excitation the absorption spectrum is characteristic of a purely low-spin species. The high-spin fraction is higher in a stationary than in a rotating sample, indicating that the high-spin contribution arises from photolysis induced by the Raman laser. This conclusion was confirmed by monitoring the absorption spectrum during laser irradiation. Photolability of the low-spin form is somewhat less than that of the CO adduct. The endogenous photolabile ligand is proposed to be the distal histidine residue, His-52. Recent picosecond absorption measurements (Jongeward et al., 1988) show that imidazole ligands in heme proteins do photodissociate but recombine in picoseconds, leading to net photostability on longer time scales. It is proposed that a fraction of the His-52 residues recombine much more slowly in CCP because of protein strain in the ligated form. This strain can also explain the anomalously rapid rate of CO binding to alkaline CCP.

We report unusual photolability of the alkaline form of reduced cytochrome *c* peroxidase (CCP) isolated from bakers' yeast or expressed in *Escherichia coli* [CCP(MI)] (Fishel et al., 1987). This enzyme catalyzes the oxidation of cytochrome *c* by hydrogen peroxide via intermediates in which the oxidizing equivalents of H_2O_2 are stored in the protein (Yonetani, 1976). Interactions between protein residues and the heme group which may be involved in assisting the required heterolytic splitting of the peroxide O–O bond have (Poulos & Kraut, 1980) been analyzed in light of X-ray crystal structures (Poulos et al., 1980; Finzel et al., 1984) and, lately, of site-directed mutagenesis experiments (Fishel et al., 1987; Goodin et al., 1987; Mauro et al., 1989). The wealth of information available about CCP makes it an invaluable testing ground for ideas for protein–cofactor interactions relevant to catalysis and to protein dynamics. Resonance Raman studies on CCP(MI) (Smulevich et al., 1988) and on selected mutants (Smulevich et al., 1988) have revealed complex and interacting forces on the heme Fe atom and on exogenous ligands, exerted via H-bond networks on both the proximal and distal sides of the heme.

In its reduced (Fe^{II}) form, CCP is high spin at low pH but low spin at high pH (Conroy et al., 1978). The same transition is found in CCP(MI), as is demonstrated by the absorption spectra, shown in Figure 1. There is a clean transformation from a single low-pH to a single high-pH form. The high-pH spectrum shows well-resolved α and β bands at 530 and 561 nm, characteristic of low-spin Fe^{II} hemes (Williams, 1951). The resonance Raman spectra of this species, however, show both low- and high-spin signals, their ratio depending on experimental conditions. The RR band which has been found to most cleanly reflect the heme spin state (Spiro, 1985) is associated with the porphyrin skeletal mode, ν_3, which is enhanced in resonance with the Soret absorption band. For high- and low-spin Fe^{II} heme complexes ν_3 is at ~1470 and ~1490 cm^{-1}, respectively. Figure 2 shows the ν_3 band region of the 413.1-nm excited RR spectrum of CCP(MI). Both low- and high-spin bands are clearly seen. Spectrum a was obtained with a defocused laser beam incident on a spinning sample, while in spectrum b the sample tube was stationary. The ratio of the low-spin (1493 cm^{-1}) to the high-spin (1471 cm^{-1}) band intensities is much lower in spectrum b. Essentially the same effect was obtained for CCP isolated from bakers' yeast. These results implicate laser photolysis as the origin of the unexpected high-spin signal. We confirmed this conclusion by demonstrating, as shown in Figure 3, that the absorption spectrum is altered by laser irradiation, in the direction expected for an increasing high-spin fraction. This experiment was carried out in an apparatus that permits absorption and RR spectra to be obtained from the same volume of sample. Laser irradiation with 55 mW of power at the sample produced successively larger changes in the absorption spectrum after 30 and 300 s; the delay in achieving a photostationary state is believed to arise from convection effects due to laser heating of the sample tube. After 300 s of irradiation, a RR spectrum similar to that of Figure 2b was obtained. At this point the photochemistry was reversible; the original absorption spectrum was recovered when the laser was turned off. [When irradiation was continued, however, (>15 min) irreversible changes were observed.] The laser photoeffect explains earlier reports of high-spin alkaline Fe^{II} species as deduced from RR spectra of CCP and mutant species from this laboratory (Smulevich et al., 1988) and others (Hashimoto et al., 1986; Dasgupta et al., 1989).

The actual degree of photolysis is not as large as the 1471/1493-cm^{-1} RR band intensity ratio suggests, however, because the molar intensity is much higher for the 1471-cm^{-1}

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* Authors to whom correspondence should be addressed.
‡ Università di Firenze.
§ University of California at San Diego.
∥ Princeton University.

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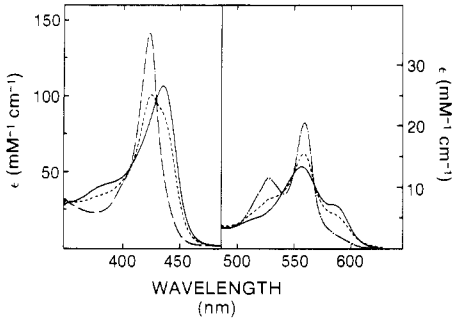

FIGURE 1: Dependence of visible and Soret region spectra of ferrous CCP(MI) on pH. The spectra were recorded in 0.1 M phosphate buffer, 23 °C (Perkin-Elmer Lambda-3B spectrophotometer with thermostated cuvette holder). Solid line, pH 6.0; dashed line, pH 7.8; broken line, pH 8.44.

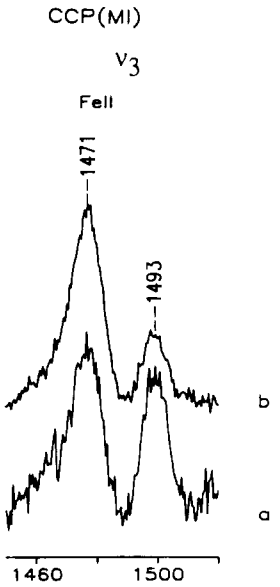

FIGURE 2: Room-temperature RR bands of ferrous CCP(MI) (~0.15 mM) at pH 8.45 in 0.1 M Tris-HCl, recorded immediately after protein crystals were dissolved in degassed buffered dithionite solution. Excitation at 413.1 nm was provided with a Coherent Kr^+ (Innova 100) laser, with 50 mW of power at the source. (a) Defocused beam and rotating NMR tube, 10 s/0.5 cm^{-1} accumulation; (b) focused beam and stationary NMR tube, 5 s/0.5 cm^{-1} accumulation interval. Back-scattered light was collected and focused into a computer-controlled double monochromator (Spex 1401) equipped with a cooled photomultiplier (RCA), as described in Smulevich et al. (1988).

band. This can be seen by examining the photoeffect in the CO adduct. Figure 4 shows the ν_4 (~1360 cm^{-1}) as well as the ν_3 region of the CCP(MI)–CO adduct RR spectra obtained under different conditions. The ν_4 band is at the same frequency, 1359 cm^{-1}, for low- and high-spin Fe^{II} hemes but is shifted to 1372 cm^{-1} by bound CO. The upshift is due to the π acid character of the CO ligand, which relieves the Fe $d_\pi \rightarrow$ porphyrin π^* back-donation, the source of the anomalously low ν_4 frequency in low-spin Fe^{II} hemes (Spiro, 1985). Spectrum a of Figure 4 was obtained with a defocused laser beam incident on a rotating sample, the same conditions as for Figure 2a. There is only a weak 1359-cm^{-1} shoulder on the 1372-cm^{-1} ν_4 band of the CO adduct, suggesting that the extent of photolysis is low. Yet, the 1471-cm^{-1} band is substantially stronger than the 1493-cm^{-1} band, showing that the high-spin/low-spin intensity ratio is much larger for ν_3 than for ν_4. No doubt, this disparity explains the misassignment of the 1471-cm^{-1} band to the CO adduct by previous workers (Dasgupta et al., 1989) although others have correctly attributed it to the photoproduct (Tsubaki et al., 1982; Uno et

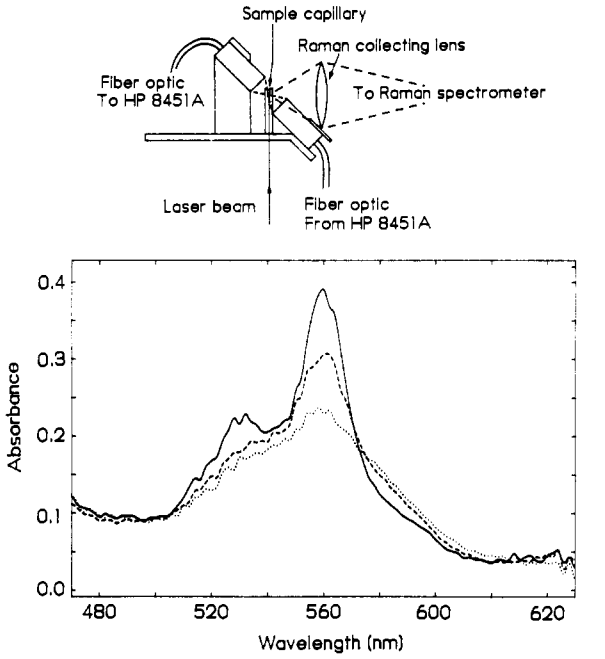

FIGURE 3: (Top) Schematic representation of the apparatus for simultaneous absorption–Raman measurements, similar to one described by Ogina and Kitagawa (1987). The fiber optic lines are coupled to a Hewlett-Packard 5451A diode array spectrophotometer. The sample was contained in a 50-μL micropipet, sealed under N_2. (Bottom) Absorption spectra of CCP(MI) (pH 8.45): solid line, before laser illumination; dashed line, after 30 s of laser illumination (55 mW on the sample); dotted line, after 300 s of laser ilumination. Each spectrum was obtained with a 30-s accumulation time.

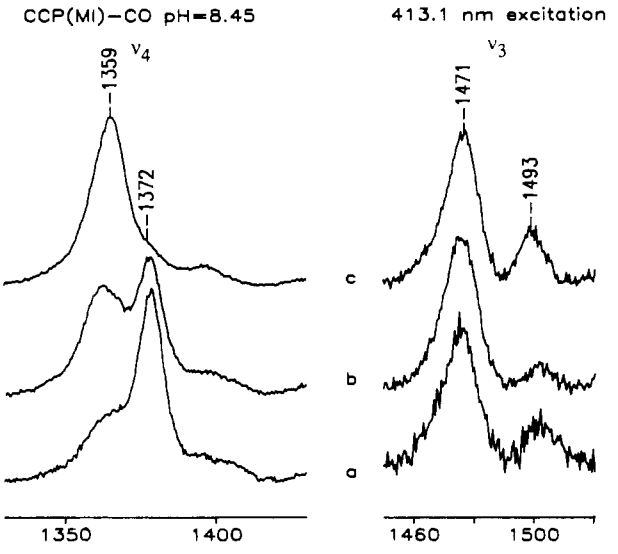

FIGURE 4: RR spectra (50 mW at the source) of CCP(MI)–CO (~0.15 mM) at pH 8.45 in 0.1 M Tris-HCl, prepared as in Figure 2 with CO (Matheson) passed over the solution for 15 min. Laser excitation and spectral aquisition are as in Figure 2. (a) Defocused beam and rotating NMR tube; 1 s/0.5 cm^{-1} accumulation interval for ν_4 and 10 s/0.5 cm^{-1} for ν_3; (b) focused beam and rotating NMR tube, 1 s/0.5 cm^{-1} accumulation interval for ν_4 and 5 s/0.5 cm^{-1} for ν_3; (c) focused beam and stationary NMR tube, 1 s/0.5 cm^{-1} accumulation interval for ν_4 and 5 s/0.5 cm^{-1} for ν_3.

al., 1987). The 1471/1493-cm^{-1} ratio is higher than it is for alkaline CCP(MI) (Figure 2a), indicating that the CO adduct is somewhat more photolabile. Spectrum b in Figure 4 was obtained with a focused beam and shows a distinct increase in both the 1359/1372- and the 1471/1493-cm^{-1} intensity ratio, consistent with greater photolysis. Spectrum c was obtained when sample rotation was stopped. Only the 1359-cm^{-1} ν_4 band is seen, indicating that CO photolysis is essentially

complete. Yet, the 1471/1493-cm^{-1} ratio is lower than in spectrum b and is the same as was observed for the stationary sample of alkaline CCP(MI) without CO, Figure 2b. We interpret this reversal as resulting from the loss of CO from the heme binding region and the restoration of the photostationary state of CO-free alkaline CCP(MI). Since it proved imposssible to obtain ν_3 or ν_4 band intensities of individual species in solutions with either zero or complete photolysis, a quantitative determination of the extent of photolysis is not possible.

While photolysis of exogenous π acid ligands like CO is well-known, the photolability of alkaline CCP, which has no exogenous ligands, comes as a surprise. It stands in marked contrast to the behavior of other low-spin Fe^{II} heme proteins, e.g., reduced cytochrome *c*, which are photostable and produce entirely low-spin RR signals (Cartling, 1987). The recent picosecond absorption study of Jongeward et al. (1988) indicates, however, that this photostability is actually a kinetic phenomenon. The endogenous ligands in cytochrome *c* and cytochrome b_5 *are* subject to photolysis, but they recombine completely in a few picoseconds, so that no effect is seen on a longer time scale. In retrospect, this finding is not altogether surprising, since the mechanism of photolysis is believed (Green et al., 1978) to involve rapid intersystem crossing from the initially elicited singlet π–π^* state of the porphyrin to a high-spin ligand field state of the Fe in which the axial ligands are only weakly bound because there is an electron in the antibonding d_{z^2} orbital. When CO is the ligand, recombination is slow, but for endogenous ligands, which are held in place by the protein, recombination is rapid and efficient. This is evidently not the case for alkaline CCP, however.

As discussed elsewhere (Smulevich et al., 1988), the likeliest sixth ligand for alkaline CCP is the imidazole ring of the distal histidine, His-52. There are no other plausible candidates among the distal residues, since an alkaline low-spin species is also found when the distal Trp-151 and Arg-48 are replaced by residues (Phe and Leu, respectively) with no potential for Fe coordination (Smulevich et al., 1988; Miller et al., 1990). While hydroxide ion is a possible ligand, it seems unlikely that hydroxide would bind to Fe^{II} at pH 8.4 or that it would form a low-spin complex (Parthasarathi et al., 1987). Moreover, a weak RR band has been detected for some alkaline CCP(MI) mutants at 200 cm^{-1} (Smulevich et al., 1988), which is the expected position of the symmetric Fe–imidazole stretch of a bis(imidazole)–heme adduct (Mitchell et al., 1987). On the other hand, the distal imidazole ring is 5.8 Å distant from the Fe atom in the crystal structure of native CCP (Finzel et al., 1984), and its binding to the Fe would require a substantial protein rearrangement. It is notable that this is not a simple deprotonation of the distal histidine; two protons are involved in the acid–alkaline transition (Conroy et al., 1978). The second proton is believed to reside on the His-181 residue, since the Gly-181 mutant shows only a single proton in its alkaline transition (Miller et al., 1990). His-181 is H bonded to a heme propionate residue (Poulos et al., 1980; Finzel et al., 1984) and influences the H-bond networks on both the proximal and distal sides of the heme, as evidenced by perturbations seen in the RR spectra of the Gly-181 mutant (G. Smulevich, M. A. Miller, and T. G. Spiro, unpublished results). Consequently its deprotonation might well trigger a protein rearrangement that permits His-52 to bind to the Fe. We note that alkaline CCP or CCP(MI) becomes noticeably less photolabile after standing for 24 h, as judged by the 1471/1493-cm^{-1} RR band intensity ratio. Apparently the altered protein conformation is stabilized by some irreversible modification. This effect may be similar to the conversion of a reversibly six-coordinate form to an irreversibly six-coordinate form described in ferric CCP from bakers' yeast (Gross & Erman, 1985; Dhaliwal & Erman, 1985; Yonetani & Anni, 1987; Dasgupta et al., 1989).

We propose that the photolability of the hexacoordinate form observed after initial exposure of CCP to alkaline conditions results from tension on the Fe–His-52 bond due to protein forces. These forces would be expected to retard the recombination rate when the Fe–His-52 bond is photolyzed, thereby accounting for the unusual photostationary state seen in the RR spectra of alkaline CCP. Picosecond absorption measurements (not shown) establish that there is a picosecond recombination process for alkaline CCP and suggest that recombination is slowed for only a small fraction of the protein molecules when the laser pulse is $\sim$1 ps in duration. This fraction builds up under continuous laser exposure to form the photostationary state. Immediately after photolysis there must be a competition between geminate recombination and a protein conformation change carrying the His-52 residue away from the heme. The rapid recombination phase can explain why the degree of photolysis seen in the cw Raman spectra is less for alkaline CCP than for its CO adduct; recombination of CO is evidently slower than that of His-52. Despite this, however, the photoproduct yield increases when the laser beam incident on the CO adduct is focused (Figure 4, spectrum b) and only decreases again when the sample is stationary (Figure 4, spectrum c). We infer that the His-52 rebinding rate is established when CO is driven from the protein by the continuous irradiation of the stationary sample but is somewhat retarded by CO molecules remaining in the vicinity of the heme at earlier times after photolysis.

On the other hand, the rate of CO rebinding to alkaline CCP(MI) is quite rapid, $\sim 10^5$ M^{-1} s^{-1} (Miller et al., 1990), much faster than would be expected for a heme protein with two firmly bound axial ligands and, indeed, considerably faster than recombination to the acidic form, in which there is no sixth ligand. We suggest that this remarkable reactivity may also be associated with the protein rearrangement required to produce the low-spin alkaline form. The resulting strain on the Fe–His-52 bond would be expected to increase the thermal dissociation rate of the His-52 ligand substantially, thereby allowing access to exogenous CO. Thus, it appears likely that protein forces control the ligand reactivity in this form of CCP.

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Common Structural Changes Accompany the Functional Inactivation of HPr by Seryl Phosphorylation or by Serine to Aspartate Substitution†

Michael Wittekind,‡ Jonathan Reizer,§ Josef Deutscher,∥ Milton H. Saier,§ and Rachel E. Klevit*,‡

Department of Biochemistry, University of Washington, Seattle, Washington 98195, Department of Biology, University of California—San Diego, La Jolla, California 92093, and Max-Planck-Institut für Systemphysiologie, Rheinlanddamm 201, D-4600 Dortmund 1, FRG

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ABSTRACT: Although many proteins are known to be regulated via reversible phosphorylation, little is known about the mechanism by which the covalent modification of seryl, threonyl, or tyrosyl residues alters the activities of the target systems. To address this question, modified versions of *Bacillus subtilus* HPr, a protein component of the bacterial phosphotransferase system, have been studied by ^{1}H NMR spectroscopy. Phosphorylation at Ser_{46} or a Ser to Asp substitution at this position inactivates HPr [Reizer, J., Sutrina, S. L., Saier, M. H., Stewart, G. C., Peterkofsky, A., & Reddy, P. (1989) *EMBO J. 8*, 2111–2120]. Two-dimensional spectra of these two modified proteins display nearly identical proton chemical shifts that differ significantly from those observed in the spectra of the unphosphorylated, wild-type protein and of functionally active HPr mutants. The results demonstrate that the functional inactivation of HPr brought about by the serine to aspartate mutation is accompanied by the same structural changes that occur when HPr is phosphorylated at Ser_{46}.

Reversible phosphorylation of proteins, catalyzed by protein kinases and phosphatases, is a widely used mechanism for regulation of cellular activities in both eukaryotes and prokaryotes (Krebs, 1985; Cozzone, 1988). There is, however, only one protein for which there is structural information for both its unphosphorylated and phosphorylated forms; glycogen phosphorylase has been shown by X-ray crystallographic techniques to undergo a conformational change upon phosphorylation of Ser_{14} (Sprang et al., 1988). These changes affect the subunit interface and influence the enzyme's response to allosteric regulators. Clearly, structural studies on other phosphorylated proteins are needed to provide an understanding of the mechanisms by which phosphorylation at a specific residue can so profoundly effect a protein's activity.

The phosphocarrier protein, HPr,[1] plays a central role in the PEP-dependent sugar transport system (PTS) in bacteria. It serves as a phosphoryl group acceptor and donor, accepting a phosphoryl moiety from PEP via phospho enzyme I to generate a phosphohistidyl intermediate and transferring it to any of a number of sugar permeases, the sugar-specific enzymes II and III of the PTS (Postma & Lengeler, 1985;

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‡University of Washington.
§University of California—San Diego.
∥Max-Planck-Institut für Systemphysiologie.

[1] Abbreviations: PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; HPr, histidine-containing phosphocarrier protein of the PTS; Ser, serine; Asp, aspartic acid; PEP, phosphoenolpyruvate; ATP, adenosine triphosphate; TSP, sodium 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$; NMR, nuclear magnetic resonance; 2D NMR, two-dimensional nuclear magnetic resonance spectroscopy; RELAY, two-dimensional relayed coherence transfer spectroscopy; NOESY, two-dimensional NOE spectroscopy.

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