that it, too, has a negative MCD effect in the Soret region. We thank Professor Ogoshi for communicating synthetic details and R. W. Lane, Jr., for helpful discussions. We also note the recent communication by Chang and Dolphin involving thiolate ligation of a porphyrin–oxygen complex: C. K. Chang and D. Dolphin, *J. Am. Chem. Soc.*, **98**, 1607 (1976).

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Chloroperoxidase. Evidence for P-450 Type Heme Environment from Magnetic Circular Dichroism Spectroscopy[1,2]

Sir:

Chloroperoxidase (CPO) and cytochrome P-450, two heme proteins with fundamentally different native activities,[3,4] have a surprising number of similar physical properties as judged by electronic absorption, electron paramagnetic resonance (EPR), and Mossbauer spectroscopy.[5] Here we present additional evidence, obtained with magnetic circular dichroism (MCD) spectroscopy, for the equivalence of their oxidized high spin, and reduced + CO heme environments. More importantly, a comparison of the MCD spectra of CPO with model heme compounds[6,7] indicates that the similarity between CPO and the P-450 cytochromes is due to thiolate ligation of the heme iron. This is contrary to the conclusion of Chiang et al.[8] based on chemical evidence that the axial ligand is *not* sulfur derived in either the native or urea-denatured protein.

Similarities between CPO and P-450 were first observed by Hollenberg and Hager[5a] who studied the absorption spectra of CPO. In addition to similarities in their oxidized and reduced states, they found that CPO, like P-450, forms a reduced + CO complex absorbing at an abnormally long wavelength (443 nm). An explanation for the unusual spectral characteristics of P-450 has been reached as a result of model heme experiments[6,7,9] which strongly implicate an axial thiolate ligand as the causal structural feature.

Figures 1–3 compare the MCD spectra[10] of CPO[11] and P-450[12-14,17-19] in their oxidized high-spin, reduced, and reduced + CO states. Similarity between the spectra of the two

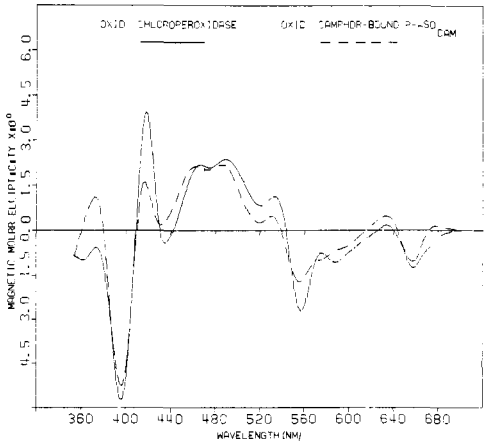

Figure 1. Magnetic circular dichroism spectra of oxidized, camphor-bound P-450$_{cam}$ at pH 7.0 (replotted from data of Vickery et al.[13b]) and oxidized chloroperoxidase at pH 3.8.

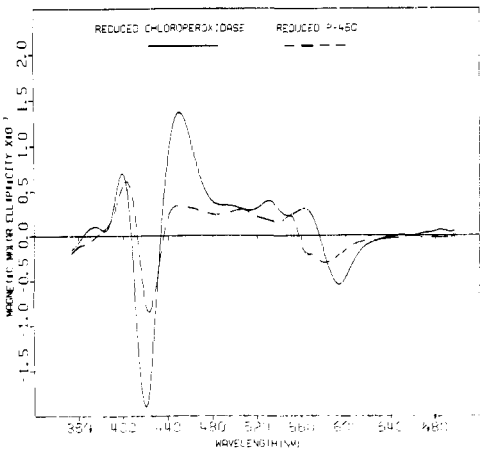

Figure 2. Magnetic circular dichroism spectra of purified, reduced P-450$_{LM2}$ at pH 7.4 and purified, reduced chloroperoxidase at pH 3.8.

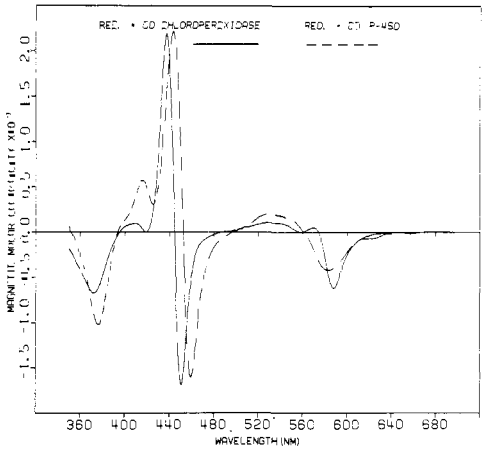

Figure 3. Magnetic circular dichroism spectra of purified, reduced + CO P-450$_{LM2}$ at pH 7.4 and purified, reduced + CO chloroperoxidase at pH 3.8.

proteins is at once apparent, particularly in the oxidized high-spin and reduced + CO states. The MCD spectrum of oxidized CPO (Figure 1) reproduces virtually all the features of oxidized high-spin P-450$_{cam}$.[12,13] Particularly noteworthy are the negative bands of nearly equal intensity in the 395-nm Soret and 660-nm charge transfer regions. While the spectra of the reduced proteins[14] (Figure 2) show gross overall resemblance, too many differences are present to conclude that their heme environments are alike. The *low temperature* Mossbauer results of Champion et al.,[5b] however, indicate that reduced CPO and P-450 do have equivalent heme environments. Whether or not it is possible to extrapolate the low temperature Mossbauer data to ambient temperatures is questionable. The inconsistencies seen in the MCD spectra may be due to differences in the spin states of the two proteins at ambient temperatures. The reduced + CO spectra are displayed in Figure 3. As discussed by Collman et al.,[7] the locations of the Soret MCD crossover points (~450 nm) for the two proteins, which correspond to the positions of their absorption maxima, may be shifted when the local polarity of their heme environment changes. The small shape differences in the 520–620-nm region are reflected in the corresponding absorption spectra.[5a,16] Aside from these minor variations, the spectra are quite similar, exhibiting equally intense MCD effects in the 450-nm Soret region, shoulders at about 420 nm, and "extra" negative bands at 370 nm. The pair of features at 450 and at 370 nm are also observed in the *hyper* spectra[20] exhibited by a number of metallo porphyrins.[21]

Our previous work comparing the MCD spectra of P-450 and models for its oxidized high-spin[6] and reduced + CO[7]

states has strongly indicated the presence of a thiolate ligand to the heme iron. Of the reduced + CO model complexes prepared by Collman and Sorrell,[9g] only the MCD spectrum[7] of the thiolate model matched that of reduced + CO P-450$_{LM2}$, reproducing each spectral attribute from the single visible band centered at 560 nm to the shoulder at 420 nm and the "extra" negative feature at 370 nm. Other models[7] showed substantial differences throughout the spectral region examined including distinct α and β bands in the visible region and lack of the "extra" negative band at 370 nm. Our work with oxidized high-spin P-450 model complexes, presented in the preceding communication,[6] provides additional spectral evidence for thiolate ligation. From that work we concluded that the appearance of a strong negative MCD band in the 395-nm Soret region would be diagnostic for thiolate ligation in high-spin ferric heme complexes. All of these results, in conjunction with the aforementioned similarity between the MCD spectra of CPO and P-450, provide strong evidence for thiolate ligation of the heme iron of CPO in its oxidized and reduced + CO states. It should be emphasized, however, that these results are based on physical measurements, whereas the contrary conclusion,[8] that a sulfur derived ligand is not present, was based on chemical studies of CPO. It is possible that the inability of Chiang et al.[8] to detect chemically a free sulfhydryl group in the native enzyme may be due to its hidden nature within the protein. To explain the lack of a free sulfhydryl group in the denatured enzyme, one needs to postulate that the thiolate ligand of CPO, when released from the iron, is sufficiently activated to react with the only other half-cystine residue in CPO[22] to form a disulfide bond. Such an explanation, if true, would remove the apparent discrepancy between chemical[8] and spectroscopic results.

The present work offers another illustration of the power and utility of MCD spectroscopy in defining certain structural aspects of this biologically important class of heme proteins.

Acknowledgments. We wish to thank Ruth Records for running some of the MCD spectra, Dr. Larry Vickery (University of California, Berkeley) for permission to replot data,[13b] and the National Institutes of Health (Grants GM 20276-02 and RG 7768) and the National Science Foundation (Grant No. B MS71-01280C) for financial assistance. One of us (J.H.D.) wishes to thank Dr. D. A. Haugen and Professor M. J. Coon for helpful discussions concerning the isolation of mammalian P-450$_{LM2}$.

References and Notes

(1) Magnetic Circular Dichroism Studies, Part 44; for Part 43 see ref 6.
(2) This work will be presented by one of us (J.H.D.) as partial fulfillment of the requirements for the Ph.D. degree in chemistry at Stanford University.
(3) J. Thomas, D. Morris, and L. P. Hager, *J. Biol. Chem.*, **245**, 3129 (1970).
(4) For reviews on P-450 see (a) I. C. Gunsalus, J. R. Meeks, J. D. Lipscomb, P. DeBrunner, and E. Munck in "Molecular Mechanisms of Oxygen Activation", O. Hayaishi, Ed., Academic Press, New York, N.Y., 1974, Chapter 14; (b) J. E. Tomazewski, D. M. Jerina, and J. W. Daly, *Annu. Rep. Med. Chem.*, **9**, 290 (1974); (c) H. A. O. Hill, A. Roder, and R. J. P. Williams, *Struct. Bonding (Berlin)*, **8**, 123 (1970).
(5) Similarities between CPO and P-450 have been extensively discussed by: (a) P. F. Hollenberg, and L. P. Hager, *J. Biol. Chem.*, **248**, 2630 (1973); (b) P. M. Champion, R. Chiang, E. Munck, P. Debrunner, and L. P. Hager, *Biochemistry*, **14**, 4159 (1975).
(6) J. H. Dawson, R. H. Holm, J. R. Trudell, G. Barth, R. E. Linder, E. Bunnenberg, C. Djerassi, and S. C. Tang, *J. Am. Chem. Soc.*, preceding paper in this issue.
(7) J. P. Collman, T. N. Sorrell, J. H. Dawson, J. R. Trudell, E. Bunnenberg, and C. Djerassi, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 6 (1976).
(8) R. Chiang, R. Makino, W. E. Spomer, and L. P. Hager, *Biochemistry*, **14**, 4166 (1975).
(9) (a) S. Koch, S. C. Tang, R. H. Holm, and R. B. Frankel, *J. Am. Chem. Soc.*, **97**, 914 (1975); (b) S. Koch, S. C. Tang, R. H. Holm, R. B. Frankel, and J. A. Ibers, *ibid.*, **97**, 916 (1975); (c) S. C. Tang, S. Koch, G. C. Papaefthymiou, S. Foner, R. B. Frankel, J. A. Ibers, and R. H. Holm, *ibid.*, **98**, 2414 (1976); (d) J. P. Collman, T. N. Sorrell, and B. M. Hoffman, *ibid.*, *97*, 913 (1975); (e) H. Ogoshi, H. Sugimoto, and Z. Yoshida, *Tetrahedron Lett.*, 2289 (1975); (f) J. O. Stern and J. Peisach, *J. Biol. Chem.*, **249**, 7495 (1974); (g) J. P. Collman and T. N. Sorrell, *J. Am. Chem. Soc.*, **97**, 4133 (1975); (h) C. K. Chang and D. Dolphin, *ibid.*, **97**, 5948 (1975).
(10) MCD measurements were made at ambient temperatures on a JASCO (Japan Spectroscopy Company) J-40 circular dichroism instrument using a 15 kg electromagnet. The protein MCD spectra have been corrected for natural circular dichroism (MCD_{obsd} = MCD + CD). All data have been normalized and are expressed in the units of molar magnetic ellipticity, $[\theta]_M$, deg cm^2 dmol^{-1} G^{-1}.
(11) Chloroperoxidase was isolated according to: D. R. Morris and L. P. Hager, *J. Biol. Chem.*, **241**, 1763 (1966). The R_z value was 1.33.
(12) Bacterially derived P-450$_{cam}$[4a] forms a homogeneous, high-spin, substrate bound species and so is used for our study of that state. The MCD spectrum of this species (Figure 1) is a replot of data presented by Vickery et al.[13b]
(13) The MCD of P-450$_{cam}$ has previously been studied by: (a) P. M. Dolinger, M. Kielczewski, J. R. Trudell, G. Barth, R. E. Linder, E. Bunnenberg, and C. Djerassi, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 399 (1974); (b) L. Vickery, A. Salmon, and K. Sauer, *Biochim. Biophys. Acta*, **386**, 87 (1975).
(14) Reduced and reduced + CO P-450 are studied with mammalian P-450$_{LM2}$[15] prepared by a modification of the method of Coon.[16] Exact details will be published elsewhere. The specific content of the P-450$_{LM2}$ was approximately 17 nmol per mg of protein.
(15) Named according to: F. P. Guengerich, D. P. Ballou, and M. J. Coon, *J. Biol. Chem.*, **250**, 7405 (1975).
(16) T. A. van der Hoeven, D. A. Haugen, and M. J. Coon, *Biochem. Biophys. Res. Commun.*, **60**, 569 (1974).
(17) The MCD of crude mammalian P-450 has previously been studied by: J. H. Dawson, P. M. Dolinger, J. R. Trudell, G. Barth, R. E. Linder, E. Bunnenberg, and C. Djerassi, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4594 (1974), and by Vickery et al.[13b]
(18) The MCD of partially purified mammalian P-450 has previously been studied by: T. Shimizu, T. Nozawa, M. Hatano, Y. Imai, and R. Sato, *Biochemistry*, **14**, 4172 (1975).
(19) The MCD of highly purified reduced + CO mammalian P-450$_{LM2}$ has previously been presented.[7]
(20) For nomenclature see: J. W. Buchler, "Porphyrins and Metalloporphyrins", K. Smith, Ed., Elsevier, Amsterdam, in press.
(21) J. H. Dawson, unpublished results.
(22) Morris and Hager[11] have previously demonstrated the presence of two half-cystine residues in CPO.

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CIDNP from Grignard Reagents Undergoing Iron-Catalyzed Halogen–Metal Exchange

Sir:

Recent reports[1] of CIDNP from Grignard reagents formed by the reaction of organohalides with magnesium have revived the view that free radicals are key intermediates in the synthesis of organomagnesium compounds.[2] While there can be no doubt that free radicals are readily formed in solutions of Grignard reagents under a variety of conditions,[2,3] the observation of CIDNP from the reagent itself has been considered to be strong evidence implicating radicals in the *primary* step of formation.[1,4]

We report here an observation which suggests an alternative interpretation of the CIDNP experiments: CIDNP of the reported type may also be produced in Grignard reagents during their iron-catalyzed reactions with organohalides *in the absence of metallic magnesium.*

In Figure 1 is shown the 0 to −1 ppm region of the proton NMR spectrum obtained before, during, and after the reaction of 0.6 M isopropylmagnesium bromide with 1.2 M *n*-butylbromide in THF catalyzed by the addition of 10^{-4} M $FeCl_2 \cdot 4H_2O$. The spectra show clearly that (a) *n*-butylmagnesium bromide is formed by halogen–metal exchange with isopropyl Grignard and, more importantly, (b) at least some of the *n*-butyl Grignard is formed via the intermediacy of *n*-butyl radicals as evidenced by the appearance of E/A CIDNP in the