Circular Dichroism and Absorption Spectra of Horse Radish Peroxidase and Sperm Whale Myoglobin in the Soret Region*

Gordon E. Willick, Gregory R. Schonbaum, and Cyril M. Kay

ABSTRACT: A study of the absorption and circular dichroism spectra of the ferri, cyanoferri, ferro, and carbon monoxyferro derivatives of horseradish peroxidase and myoglobin has been made. The results indicate that the heme environment in these proteins differs, and suggest that the hemoprotein conformation remains relatively invariant in myoglobin but not in horseradish peroxidase derivatives. Thus, in contrast to the corresponding myoglobin compounds, horseradish peroxidase loses most of the heme-associated optical activity on reduction, but gains a strong, complex circular dichroism spectrum on formation of the carbon monoxy derivative. An explanation for this behavior is advanced, based on the assumption that a major part of the Soret region rotatory power arises from the interaction of a nearly degenerate x, y polarized π–π^* in plane heme transition with nearby protein chromophores. It is concluded that horseradish peroxidase undergoes local conformational change with respect to the hemoprotein binding site on reduction from the ferri to the ferro form.

In recent years extensive studies have been carried out on the structure and function of myoglobin and its derivatives. These have included studies on the optical rotatory dispersion (Samejima and Yang, 1964; Urnes, 1963), circular dichroism (Beychok, 1967), and nuclear magnetic resonance (Wüthrich *et al.*, 1968). In particular, the crystallographic work of Nobbs *et al.* (1966) led to an intricate knowledge of the immediate environment of the heme group. Such information, which is an essential prerequisite in the interpretation of the functional diversity among hemoproteins, is not available on HRP.[1] However, both Mb and HRP share in common at least one feature, namely, the same prosthetic group, ferriprotoporphyrin IX. It is therefore of interest to compare parameters which reflect its interaction with its immediate environment.

One such property is the optical activity associated with the heme transitions. Since free heme is optically inactive (Urry, 1965), the optical activity associated with the heme transitions in hemoproteins is a direct result of the binding of heme to apoprotein. In addition, since both HRP and Mb contain only one heme per molecule, the possibility of heme–heme interactions complicating the absorption and circular dichroism spectra is eliminated. In the present study, we compare the absorption and circular dichroism spectra of ferri- and ferromyoglobin and HRP, and the cyanide and carbon monoxy derivatives of the oxidized and reduced forms, respectively. According to Moffitt and Moscowitz (1959), strong transitions ($\epsilon_{\max}$ greater than about 1000) are expected to have their absorption and associated ellipticity curves centered at the same wavelength and, in addition, they are expected to have the same shape. The transitions considered here, with extinction coefficients greater than 20,000, easily meet the above requirement for a strong transition. Since the ellipticities, if any, associated with the absorptions may be either positive or negative, it is often possible to gain information as to the number and locations of the electronic transitions through a comparison of the absorption and circular dichroism spectra. In order to assist in this comparison, it is helpful to resolve the spectra in terms of some sort of assumed functional dependence of the individual bands. It is commonly assumed that an individual band can be written as a symmetric gaussian about the band maximum (Moscowitz, 1960a). This functional dependence has also been assumed here, in order to provide a basis for analysis. However, it should be remembered that the use of the symmetric gaussians has no theoretical significance and is simply a matter of analytical convenience.

Materials and Methods

Hemoproteins. Crystalline, salt-free sperm whale ferriMb was obtained from the Mann Chemical Co. The sample contained 3% oxymyoglobin. The concentration of ferriMb was calculated from the molar absorptivities ϵ_{409} 1.68×10^5, ϵ_{634} 3.67×10^3, and ϵ_{280} 3.34×10^4, which in turn were based on an estimation of the hemin content according to Paul *et al.* (1953).

HRP was obtained electrophoretically pure from Worthington Biochemical Corp., Freehold, N. J. The heme content was analyzed in the same way as the ferriMb. The extinction coefficient obtained at the Soret band maximum was ϵ_{403} 1.02×10^5 M^{-1} cm^{-1}. The ratio of absorptivities, *RZ*, defined as $\epsilon_{403}/\epsilon_{275}$, was 3.25.

Buffers. Unless otherwise noted, all spectra were obtained in a potassium dihydrogen phosphate–sodium monohydrogen phosphate buffer with an ionic strength of 0.05 M and pH 7.0 at 20°.

Derivatives. FerriHRP was converted into cyanoferriHRP by the addition of potassium cyanide to a concentration of 0.3×10^{-3} M. For cyanoferriMb the final concentration of potassium cyanide was 10^{-3} M.

FerroHRP and ferroMb were prepared in oxygen-free buffers by adding a slight excess of sodium dithionite to their

---

* From the Department of Biochemistry, University of Alberta Medical School, Edmonton, Canada. *Received April 23, 1969.* This study was supported by grants from the Medical Research Council of Canada, the Life Insurance Medical Research Fund, and the Canadian Muscular Dystrophy Association.

[1] Abbreviations used are: HRP, horseradish peroxidase; Mb, sperm whale myoglobin; DBM, Debye–Bohr magneton.

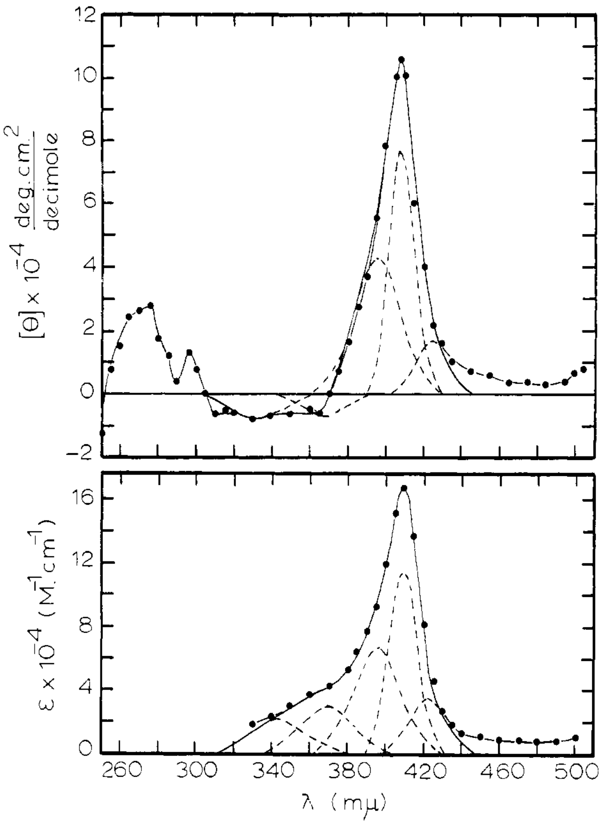

FIGURE 1: Circular dichroism (upper) and absorption (lower) spectra of ferriMb. For details, see text.

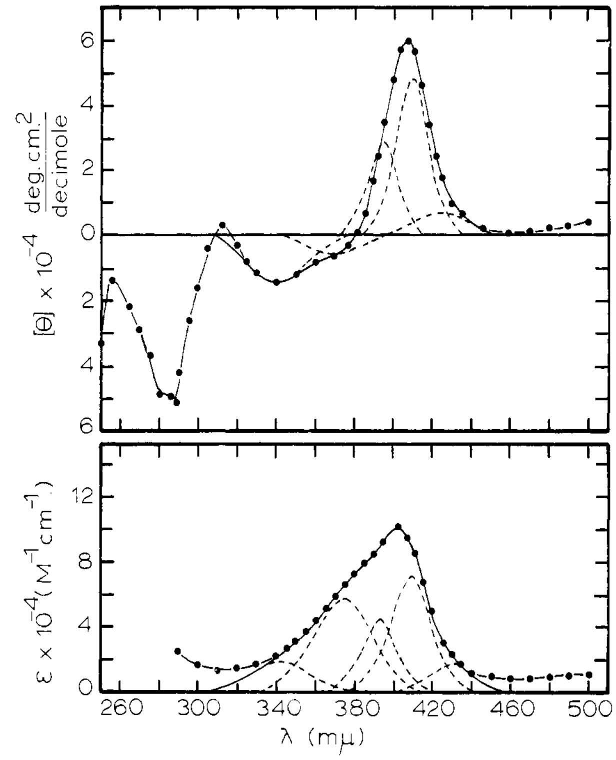

FIGURE 2: Circular dichroism (upper) and absorption (lower) spectra of ferriHRP. For details, see text.

respective ferri compounds. The carbon monoxyferro derivatives were prepared by passing a gentle stream of carbon monoxide for 2 min through the solutions of the ferro derivatives. The carbon monoxide was washed with a 5% solution of alkaline pyrogallol to remove any residual oxygen.

Spectroscopy. Circular dichroism measurements were carried out with a Cary 60 recording spectropolarimeter equipped with circular dichroism attachment, at a room ambient temperature of about 20°. Absorption spectra were obtained with a Cary 14 recording spectrometer.

Centrifugation. Sedimentation coefficients were measured with a Beckman Model E ultracentrifuge using the schlieren optical system.

Curve Resolving. Curve resolving was carried out with a five-channel DuPont curve analyzer, calibrated to a standard Gaussian curve. After an initial rough examination of all 16 spectra, it was found that a minimum of five gaussians were necessary to describe the spectra in the 340–440-mμ region. Thenceforth, the corresponding absorption and circular dichroism spectra were compared, with an individual circular dichroism band positioned no further than 5 mμ away from its corresponding absorption band. Corresponding band widths were maintained within 20%, and usually much less.

Results and Discussion

The absorption and circular dichroism spectra of ferri-, cyanoferri-, ferro-, and carbon monoxyferroHRP and myoglobin in the wavelength range 250–500 mμ are presented in Figures 1–8. It is frequently assumed that the absorption or circular dichroism spectrum of a single electronic transition can be represented as a single function gaussian in wavelength, λ, about λ_k or λ_k^0 the respective centers of the Gaussians. Such an approximation is not necessarily satisfactory in every case (Moscowitz, 1960a), and consequently any analysis such as this should be viewed with caution.

The absorption and circular dichroism of ferriMb and ferriHRP are presented in Figures 1 and 2. (Regarding the HRP used in these studies, it should be made clear that its isoenzyme composition has not been strictly defined, but it appears to represent predominantly isoenzyme C (Strickland *et al.*, 1968). These authors have shown that isoenzymes C and A1 of ferriHRP have very similar absorption and circular dichroism spectra in the Soret region.) The gaussian resolutions are indicated by the dashed lines. The experimental data are represented by the solid circles, whereas the solid line shows the fit of the sum of the gaussians to the data. A broken line has been placed through the points in the regions which were not resolved. The asymmetry of the ferriMb absorption and circular dichroism spectra necessitates the use of at least two relatively large gaussians; these bands have been placed at 396 and 409 mμ (Figure 1). If these postulated bands actually exist and are not simply artifacts of the gaussian approximation used for the analysis, they may be vibronic components of the B band. (We shall use the term B band to describe that portion of the Soret region presumably arising from a π–π^* transition of the porphyrin ring; Zerner *et al.*, 1966.) The postulated B components are split by about 15 mμ, too wide to be due to a degeneracy from the B_x and B_y in plane dipole transitions of the porphyrin ring. Eaton and Hoch-

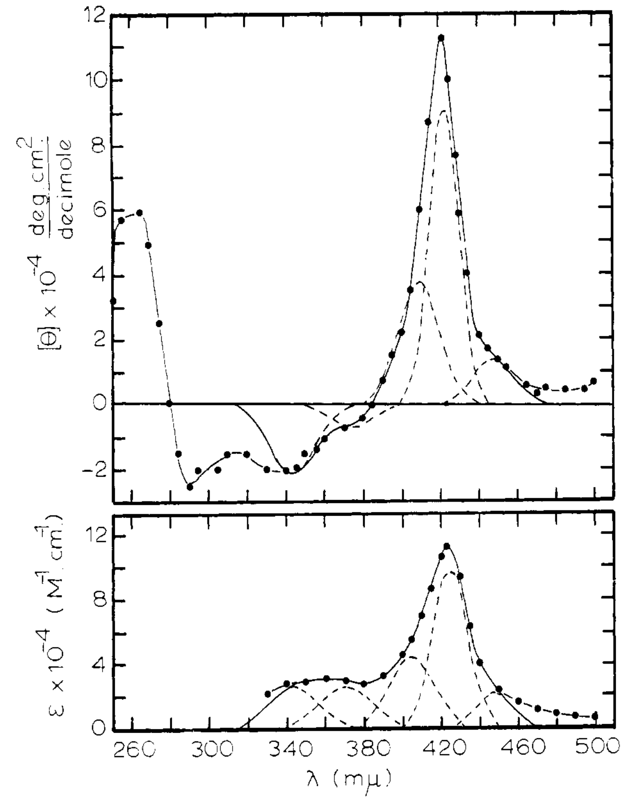

FIGURE 3: Circular dichroism (upper) and absorption (lower) spectra of cyanoferriMb. For details, see text.

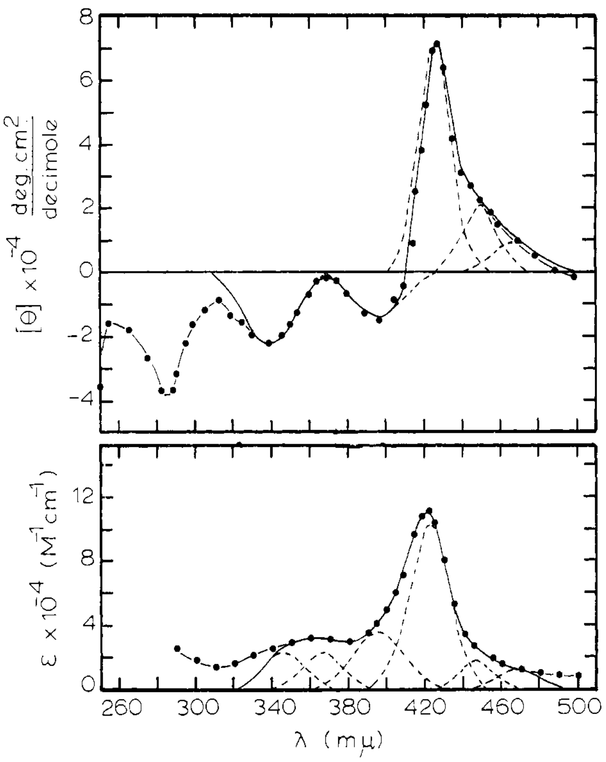

FIGURE 4: Circular dichroism (upper) and absorption (lower) spectra of cyanoferroHRP. For details, see text.

strasser (1968) have demonstrated the splitting of these components in acid ferriMb by means of the single crystal polarization ratio spectrum. However, they believe the splitting is less than 5 mμ.

Both ferriMb and ferriHRP exhibit weak ellipticities at about 360 mμ, thus suggesting the presence of still another band. This band has been placed at 372 mμ in ferriHRP and corresponds to a shoulder in the absorption spectrum, and as a weaker band at 368 mμ in ferriMb. It seems most reasonable to assign the band at 370 mμ to a charge-transfer transition, since no further π–π^* transitions are predicted to occur in this region (Zerner *et al.*, 1966). It is interesting to note that the increase in the 372-mμ band in ferriHRP as compared with ferriMb corresponds to a decrease in the supposed B band components, thus suggesting these transitions may in some way be coupled. The weaker bands at wavelengths higher than the B bands in these and other derivatives may also be charge-transfer bands, but it should be clear, that, if present, their assignment rests mainly on the gaussian approximations used in the resolution.

An additional band has been placed at about 340 mμ in all spectra where information was obtained in this region. Although, with the exception of the cyano derivatives, there is little indication of its presence in the absorption spectra, it is clearly seen as an almost isolated band in most of the circular dichroism spectra. This band is presumably the N band, a π–π^* heme transition, which is predicted theoretically to lie in this region (Zerner *et al.*, 1966).

The cyanide derivatives of ferriMb and ferriHRP show definite differences in the circular dichroism, although their absorption spectra are very similar (Figures 3 and 4). CyanoferriHRP has a clearly discernible shoulder at about 445 mμ, thus providing some tangible evidence for the inclusion of the band at 448 mμ in the absorption and circular dichroism resolutions of these derivatives. HRP and Mb also differ in that the circular dichroism spectrum of HRP has a pronounced minimum at about 397 mμ, whereas the ellipticity of Mb is positive at this wavelength. This observation may be interpreted in terms of the contribution of opposite partial ellipticities near 400 mμ, positive for Mb and negative for HRP.

While the circular dichroism spectra of the two ferri derivatives of HRP and Mb share many similar features, those of the reduced derivatives show major differences (Figures 5 and 6). FerroMb gave a rotational strength in the region of the Soret band which was even larger in relation to the oscillator strength than in the case of ferriMb. On the other hand, ferroHRP, which has a very similar absorption spectrum to ferroMb, showed an apparent loss of optical activity in the Soret region. In terms of the gaussian resolutions, it is possible to reconcile this with a complete loss of optical activity associated with the principal absorption band in the 435–440-mμ region, along with a large reduction in the activity of a band at about 420 mμ. This behavior cannot be due to artifacts such as hemin degradation or protein denaturation since the absorption spectrum, in the visible and Soret region, are typically those of ferroHRP. Furthermore, following autoxidation of ferroHRP, the circular dichroism spectrum was reexamined and found to be identical, within experimental error, with that of ferriHRP untreated with sodium dithionite.

The absorption and circular dichroism spectra of the carbon monoxy derivatives of ferroMb and ferroHRP are given in Figures 7 and 8. The complex circular dichroism spectrum

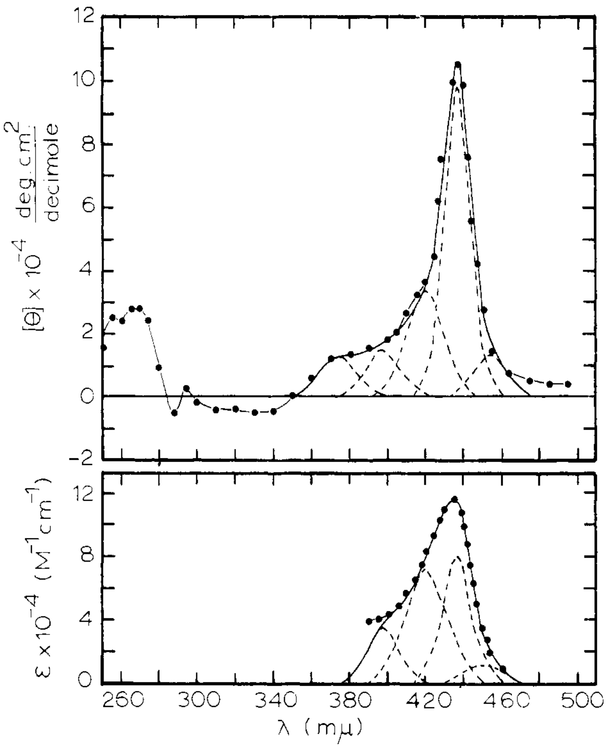

FIGURE 5: Circular dichroism (upper) and absorption (lower) spectra of ferroMb. For details, see text.

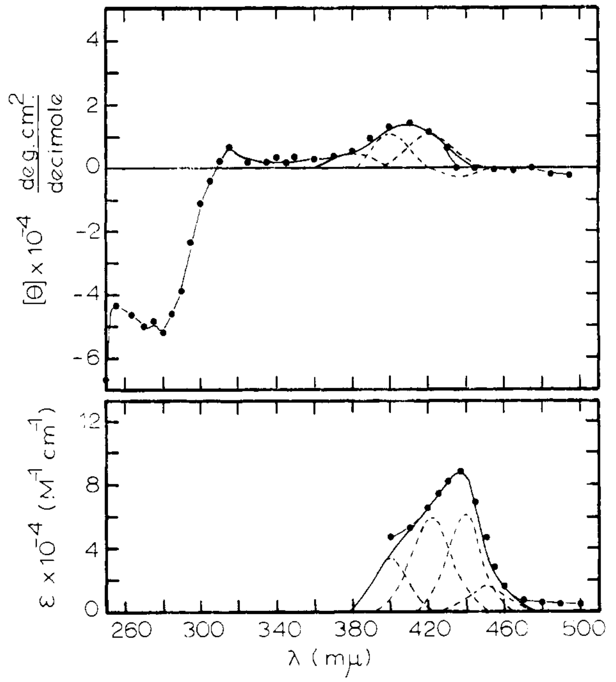

FIGURE 6: Circular dichroism (upper) and absorption (lower) spectra of ferroHRP. For details, see text.

of carbon monoxyferroHRP can only be explained by splitting the major band of the Soret region into two components and giving the component with higher energy a negative ellipticity. The magnitude of the individual rotational strengths in such a "couple" cannot be uniquely specified (Schellman, 1968). The absorption and circular dichroism spectra of carbon monoxyferroMb suggest that only one major band is present in the Soret region. We have also split the carbon monoxyferroMb band into two components separated by about 8 mμ, in order to demonstrate that ferroMb can be treated in a manner consistent to the treatment of carbon monoxyferroHRP. However, there is no direct evidence to show that the carbon monoxyferroMb band must be so split.

Although the resolution of the spectra in terms of the gaussian bands may be interpreted in terms of the number and location of the heme transitions in these proteins, it should be again emphasized that they are not necessarily unique. This is particularly the case where rotational couples may be present, as, for example, in carbon monoxyferroHRP. The circular dichroism spectrum, where there is the possibility of oppositely signed rotational strengths, provides the principle justification for the positioning of the gaussians. It was found that a consistent treatment of the spectra examined required a minimum of five gaussians in the 340–440-mμ region. It may be noteworthy that circular dichroism studies of cytochrome C (Vinogradov and Zand, 1968) and a heme octapeptide from cytochrome C (Urry and Pettegrew, 1967) both suggest at least five optically active transitions in the Soret region. However, the porphyrin moiety in cytochrome C and its heme octapeptide is a biscysteine adduct of protoporphyrin, and this could lead to a richer spectrum of charge-transfer transitions.

Heme–heme exciton interactions have been used to explain a complex spectrum, such as that seen in carbon monoxyferroHRP, in the case of the heme undecapeptide and heme octapeptide systems from horse heart cytochrome C (Urry, 1967; Urry and Pettegrew, 1967) and ferrocytochrome oxidase (Urry *et al.*, 1967). If the splitting was sufficiently small in the case of ferroHRP, the opposing ellipticities of the same magnitude might approximately cancel, whereas the carbon monoxy ligand field might increase the splitting and give rise to the couplet-type spectrum observed. Therefore, it was decided to investigate the state of aggregation of ferroHRP. Although ferriHRP contains only one heme per mole and is known to be monomeric under the conditions used in these experiments, there is no corresponding information on the state of aggregation of ferroHRP. Consequently, the sedimentation coefficient of HRP was measured before and after the addition of an excess of sodium dithionite. At a concentration of 1.9 mg/ml, the values for $s_{20,w}$ for the oxidized and reduced forms were 3.70 and 3.67 S, respectively. Thus, there is no evidence for the dimerization of ferroHRP, and heme–heme interactions cannot explain the circular dichroism spectra of ferro- and carbon monoxyferroHRP. By analogy, the complex spectrum obtained with reduced cytochrome oxidase is not necessarily due to heme–heme interaction as previously postulated (Urry *et al.*, 1967). An alternative explanation for these results will be considered shortly.

Our results therefore indicate that a conformational change in the hemoprotein binding takes place on the reduction of ferriHRP to ferroHRP. Two mechanisms may be envisaged by which the heme becomes optically active on binding to the protein. In one case, the planar structure of the heme is distorted on binding to the apoprotein, thus destroying the basic symmetry of the heme and making it intrinsically optically active. In the other case, the heme can be considered to become optically active as result of the asymmetric perturbation

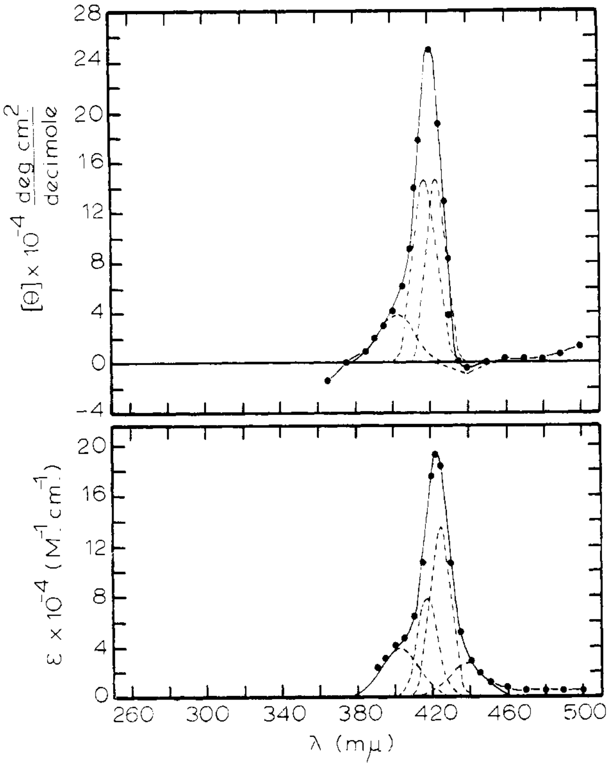

FIGURE 7: Circular dichroism (upper) and absorption (lower) spectra of carbon monoxyferroMb. For details, see text.

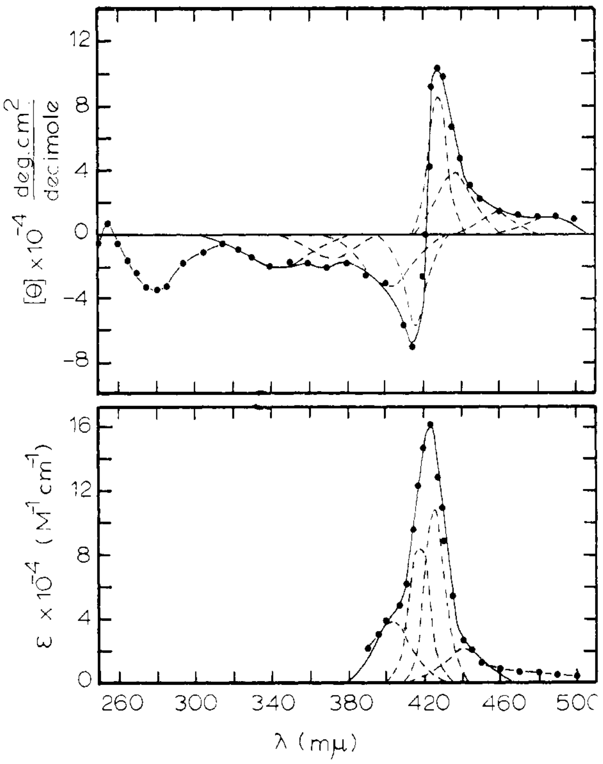

FIGURE 8: Circular dichroism (upper) and absorption (lower) spectra of carbon monoxyferroHRP. For details, see text.

of the heme transitions by the surrounding protein. Regardless of the mechanism, however, the gross change in the circular dichroism of HRP on reduction suggests that a conformational change does take place. No measurable change occurred in the negative ellipticity minima at 207 and 220 mμ. Since this portion of the spectrum is associated with the secondary structure of the protein (Holzwarth and Doty, 1965), no measurable change has occurred in the protein which would give rise to an alteration in this secondary structure. Thus, it appears likely that the change in the Soret circular dichroism on reduction of ferriHRP to ferroHRP results from a hemoprotein conformational change restricted to the immediate vicinity of the heme.

A recent study of Hsu and Woody (1969) on the origin of the optical activity of heme transitions in myoglobin may also provide a possible explanation for the circular dichroism spectra of ferroHRP and carbon monoxyferroHRP. They have found that a positive rotational strength for the B band of about 0.3 DBM is obtained by considering the coupling of the B_x and B_y components with the aromatic side chains of the apoprotein. The calculations give a positive rotational strength for the B_x component of about 1.3 DBM, greater by 0.3 DBM than the negative rotational strength of the B_y component. Since the splitting is small, and the magnitudes of the opposing transitions differ substantially, one expects to observe only an apparent positive rotational strength from the sum of the two (Wellman *et al.*, 1965). Contributions to the rotational strength from alkyl side chains vicinal to the heme and the polypeptide backbone were found to be minor. The net rotational strength observed will depend upon the relative geometry of the heme and the protein. In addition, the rotational strengths of the individual B_x and B_y components strongly depend upon the orientations of these components in the heme plane, although the net rotational strength will be independent of this factor. The orientation of the B_x and B_y components is likely to be sensitive to the presence of a ligand, such as CO, in the sixth coordination position. For a given small splitting, the observed circular dichroism spectra of ferroHRP and carbon monoxyferroHRP can be generated by an appropriate choice of rotational strengths for the B_x and B_y components. If the major difference in the circular dichroism spectra of these two species is due solely to a change in the orientation of the B_x and B_y components on addition of the carbon monoxy ligand, the net rotational strength should remain the same as in ferroHRP.

We may calculate the rotational strengths most easily from the gaussian resolutions. To a good approximation, the rotational strength, in DBM, of the gaussian k is given by (Moscowitz, 1960b)

$$R_k = 0.75 \times 10^{-4} \frac{(\pi^{1/2}[\theta]_k{}^0\ \Delta_k{}^0)}{\lambda_k{}^0} \qquad (1)$$

where $[\theta]_k{}^0$ is the ellipticity at the wavelength of the band maximum, $\lambda_k{}^0$, and $\Delta_k{}^0$ is the half-band width where $[\theta]_k$ is e^{-1} times $[\theta]_k{}^0$. The total rotational strength for the Soret region can simply be found by summing the resolving gaussians.

The total rotational strength for the Mb Soret region was calculated to be 0.45 DBM, whereas the rotational strength for the gaussian band at 408 mμ is 0.24 DBM. Since the Soret region is almost certainly mixed with some transitions other than the B transition used by Hsu and Woody (1969) in their calculations, 0.45 DBM can be considered an upper limit to be compared with these calculations. Conversely, 0.24 DBM could be considered to be a lower limit. Consequently, the value of 0.3 DBM obtained by Hsu and Woody (1969) really agrees quite well with what we infer as the experimental limits.

We may now consider the reduced HRP case. The net rotation in the Soret region for ferroHRP is 0.1 DBM, and for carbon monoxyferroHRP 0.05 DBM. The two bands in carbon monoxyferroHRP at 417 and 428 mμ are split by 11 mμ. Although this is high for the polarized splitting of the B band, there is considerable leeway in the placing of these two opposing bands in the spectrum. The total rotation of these two bands is 0.03 DBM. Thus the sum is approximately the same in both cases, and the data are consistent with the preceding theory on the origin of the complex reduced HRP circular dichroism spectra. In the case of the other derivatives studied, any of the principle gaussians could have been replaced by closely split pairs of gaussians, but there was no direct evidence for this from the circular dichroism spectra in these cases.

Although we have advanced the preceding as a possible explanation for the experimental observations on the circular dichroism of HRP, we do not wish to imply that it is the only possible one. If the heme were to become nonplanar on binding to either of the apoproteins, optical activity could result. Nonplanarity of the protoporphyrin ring has not been reported, although the iron has been estimated to be about 0.30 Å out of the plane of the ring in ferrimyoglobin (Kendrew *et al.*, 1960). However, the existing X-ray crystallographic data may not rule out heme distortion on the binding of heme to apoprotein (Hoard, 1968). The porphine skeleton has been found to be readily deformable by environmental stresses into a ruffled or domed configuration (Hoard, 1968). If the protoporphyrin were deformed so as to become intrinsically asymmetric on the binding of heme to apoMb and apoHRP the resulting optical activity could be explained. Loss of activity in ferroHRP would then imply that the conformational hemoprotein change restored planarity to the heme. It is possible that the complex spectrum observed with carbon monoxyferroHRP could result from a restoration of a different heme nonplanarity on the binding of carbon monoxide to ferroHRP. With either interpretation of these results, the same conclusion—that there is a conformational change on the reduction of ferriHRP—is reached. However, in the case of the latter explanation, formation of the carbonmonoxide derivative results in a further conformational change, while in the case of the former it does not.

We found no evidence for a change in conformation on the reduction of ferriMb to ferroMb. Our conclusion is therefore the same as that of Samejima and Yang (1964), who found no evidence for any conformational change on reduction of ferriMb from optical rotatory dispersion studies. X-Ray crystallographic studies, to 2.8-Å resolution, have also indicated no conformational change on reduction (Nobbs *et al.*, 1966). On the other hand, the absorption and circular dichroism spectra of ferriHRP and its cyanide derivative show sufficient differences as compared with the corresponding myoglobin spectra to warrant the conclusion that the heme environment differs in the two cases.

However, it may be that we are placing too much emphasis on these observed differences in the absorption and circular dichroism spectra of ferriHRP and ferriMb. Cytochrome C peroxidase, which has a very similar absorption spectrum to ferriHRP (Yonetani, 1965), shows a negative Cotton effect in the Soret region.[2] In view of this, and in the light of the preceding discussion based on the work of Hsu and Woody (1969), it may be that it is the *similarities*, rather than the differences in the Soret Cotton effects of ferriHRP and ferriMb, which are the more striking.

Acknowledgments

The authors thank Dr. Robert W. Woody for some very helpful discussions and also for providing us with a copy of his manuscript prior to publication. They also acknowledge the very competent technical assistance of Mr. K. Oikawa.

References

Beychok, S. (1967), *in* Poly-α-amino Acids, Fasman G., Ed., New York, N. Y., Marcel Dekker, p 332.

Eaton, W. A., and Hochstrasser, R. M. (1968), *J. Chem. Phys. 49*, 985.

Hoard, J. L. (1968), *in* Structural Chemistry and Molecular Biology, Rich, A., and Davidson, N., Ed., San Francisco, Calif., Freeman, p 573.

Holzwarth, G., and Doty, P. (1965), *J. Am. Chem. Soc. 87*, 218.

Hsu, M.-C., and Woody, R. W. (1969), *J. Am. Chem. Soc. 91*, 3679.

Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C., and Shore, V. C. (1960), *Nature 185*, 422.

Moffitt, W., and Moscowitz, A. (1959), *J. Chem. Phys. 30*, 648.

Moscowitz, A. (1960a), *Rev. Modern Phys. 32*, 440.

Moscowitz, A. (1960b), *in* Optical Rotatory Dispersion, Djerassi, C., Ed., New York, N. Y., McGraw-Hill, p 165.

Nobbs, C. L., Watson, H. C., and Kendrew, J. C. (1966), *Nature 209*, 339.

Paul, K. G., Theorell, H., and Åkeson, A. (1953), *Acta Chem. Scand. 7*, 1284.

Samejima, T., and Yang, J. T. (1964), *J. Mol. Biol. 8*, 863.

Schellman, J. A. (1968), *Accounts Chem. Res. 1*, 144.

Strickland, E. H., Kay, C., Shannon, L. M., and Horwitz, J. (1968), *J. Biol. Chem. 243*, 3560.

Urnes, P. J. (1963), Ph.D. Dissertation, Harvard University, Cambridge, Mass.

Urry, D. W. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 640.

Urry, D. W. (1967), *J. Am. Chem. Soc. 89*, 4190.

Urry, D. W., and Pettegrew, J. W. (1967), *J. Am. Chem. Soc. 89*, 5276.

Urry, D. W., Wainio, W. W., and Grebner, D. (1967), *Biochem. Biophys. Res. Commun. 27*, 625.

Vinogradov, S., and Zand, R. (1968), *Arch. Biochem. Biophys. 125*, 902.

Wellman, K. M., Laur, P. H. A., Briggs, W. S., Moscowitz, A., and Djerassi, C. (1965), *J. Am. Chem. Soc. 87*, 66.

Wüthrich, K., Shulman, R. C., and Peisach, J. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 373.

Yonetani, T. (1965), *J. Biol. Chem. 240*, 4509.

Zerner, M., Gouterman, M., and Kobayashi, H. (1966), *Theoret. Chim. Acta (Berlin) 6*, 363.

---

[2] G. E. Willick, G. R. Schonbaum, and C. M. Kay, unpublished data.