The Heme Chromophore in the Ultraviolet

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SUMMARY

Absorption, optical rotatory dispersion, and circular dichroism data are presented for the oxidized and reduced states of the heme undecapeptide of cytochrome c. The absence of aromatic residues, disulfides, and ionizable sulfhydryl groups allows unambiguous determination of the heme absorptions to 240 mp. As the electronic transitions within the heme are made optically active by the covalently attached peptide, it is possible to detect bands not readily resolved from absorption data. An absorption maximum is seen at 277 mp which is labeled the c hemochromogen band. In the presence of imidazole, optically active bands are found in the ferroheme undecapeptide with extrema at 263 mp, 278 mp, 317 mp, and 353 mp. The ferriheme undecapeptide exhibits a positive circular dichroism band at 253 mp and a broad negative extremum in the 320- to 360-mp wavelength range. These bands, including those which are found in the wavelength range normally reserved to the aromatic amino acids, are due to the heme chromophore. At shorter wavelengths it is shown that the absorption at 190 mp cannot be adequately accounted for by the peptide moiety and the carboxyls of the propionic acid heme substituents, that is, 25 to 50% of the absorption at 190 mp is due to heme, the coordinated imidazole, and the thiol other groups. The circular dichroism in the peptide region is dependent on the oxidative state and indicates changes directly involving the heme moiety either electronically or sterically.

The bands observed in the heme peptides are briefly compared to qualitatively similar bands in cytochrome c, myoglobin, and hemoglobin. The distinctive feature in the circular dichroism of the heme proteins is the presence of local bands which correspond to vibrational bands present in the aromatic amino acids. The vibronic bands should allow assessment of environmental changes in the corresponding amino acids.

The heme proteins exhibit absorption and optical rotation changes in the ultraviolet which are dependent on their state of oxidation or on the nature of bound ligands. The optical rotatory dispersion curves of ferri- and ferrocytochrome c show marked differences in the 350 to 185 mp range (1-4). ORD data of myoglobin and hemoglobin also display rotation in the ultraviolet that is dependent on oxidation state and ligand (5, 6). Before these differences can be interpreted in terms of changes in helical contents, or changes in the environment of a particular aromatic amino acid, it is necessary to characterize the heme chromophore over this accessible range. Difference absorbance studies have recently been used to assess different states of heme proteins (7). Interpretations of absorption changes are made difficult by wavelength shifts and modified intensities which complicate attempts to ascribe the alterations to given chromophoric groups and thereby confound attempts to interpret the information in terms of molecular structure. In the 350- to 185-mp wavelength range of heme proteins, electronic transitions may be grouped into three sources: transitions in amino acids with side chain chromophores, such as phenylalanine, tyrosine, tryptophan, imidazole, cysteine and cystine, transitions in the peptide chromophore, and the heme transitions. In studying heme proteins one would like to assign an absorption or circular dichroism band to a particular group such that modifications in the extremum may be interpreted in terms of variations in the environment of that residue. In particular, a CD band in the vicinity of 260 mp is observed which is greatly increased on oxygenation of deoxymyoglobin or deoxyhemoglobin. Can such a change in rotational strength as a result of oxygenation be interpreted as a change in the environment of a phenylalanine residue?

The peptic digestion product of cytochrome c results in a heme undecapeptide in which there are no aromatic amino acids except the imidazole of histidine (8, 9). The only group in the heme undecapeptide with electronic transitions in the 350- to 340-mp range is the heme moiety. Thus, absorption and CD bands observed in this wavelength range may be assigned to the heme chromophore. The absence of a heme band in the heme peptide does not preclude its presence in heme proteins; however, the presence of a band in the heme peptide which corresponds to a band in this range in hemoproteins is most reasonably taken as having its origin in the heme group. The presence of such bands complicates assignment of bands to aromatic residues in the hemoproteins. In this communication we show the presence of an c hemochromogen absorption band at 277 mp, and circular dichroism extrema at 320 mp, 278 mp, and 263 mp in the ferro-

1 The abbreviations used are: ORD, optical rotatory dispersion; CD, circular dichroism.
Heme Chromophore in Ultraviolet

EXPERIMENTAL PROCEDURE

The heme undecapeptide was prepared as outlined by Harbury and Loach (10). The amino acid composition was determined after digestion in concentrated HCl-glacial acetic acid (1:1) at 100° overnight in a sealed tube. The analysis agreed very well with the reported values (10) and known sequence (11). Reduction was achieved by addition of dithionite. The absorption curve of the ferroheme undecapeptide was determined after reduction by dithionite and separation from reductant on a G-10 over G-25 Sephadex column. The reduction and purification were carried out in a controlled atmosphere glove box. The reductant-free ferroheme peptide was collected in spectrophotometer cells of varying path length. The cells were sealed with paraffin before removal from the oxygen-free atmosphere of the glove box and the absorption and optical rotatory dispersion curves were run immediately. The samples were then allowed to air oxidize in the refrigerator and the curves were again run to determine the absorption and optical rotation of the oxidized form. The samples were buffered with sodium tetraborate at pH 7. Concentrations were determined on the basis of an ε_{280} of 160 (12). Human hemoglobin was prepared from freshly drawn blood. The raw lysate was run first as the oxyhemoglobin and then reduced with dithionite. The parallel curves were determined, respectively, with the Cary model 14 spectrophotometer, the Cary model 60 spectropolarimeter, and a prototype circular dichroism attachment built by Cary Instruments for the model 60. A spectral bandwidth of 15 A was used for the optical rotation measurements. Unless otherwise stated, the temperature was 27°. Scan speeds, pen periods, and time constants were chosen to maintain adequate response time and to allow adequate signal to noise ratios. The prototype CD unit was calibrated by using the Cary model 1401 circular dichroism attachment for the model 14. The standard used was an aqueous solution of d-10-erythorol sulfo acid (J. T. Baker, Lot No. 9-36) with an ε_{190} of 2.2 at 290 μ. The conversion factor was 2.67 × 10^{-2} ΔOD per degree. pH measurements were made on a Radiometer model 5SE pH meter. Cell path lengths were calibrated with solutions of chromate in 0.05N KOH.

RESULTS AND DISCUSSION

Absorption of Heme Peptide—The ferroheme undecapeptide exhibits a characteristic hemochromogen spectrum with the same α:β ratio obtained for ferrocytochrome c. It is to be expected that the heme chromophore of the heme undecapeptide bears important similarity to that of cytochrome c over the accessible range. As the heme undecapeptide contains no phenylalanine, tyrosine, tryptophan, cystine, or ionizable sulphydryl, it is possible to determine the hemochromogen spectrum from 600 μ to 240 μ with assurance that all the absorption bands involve the heme moiety. Fig. 1 shows the absorption spectra of oxidized and reduced heme peptide in the absence of reductant or oxidant and their products. A band at 277 μ is clearly distinguishable in the reduced form. This absorption is labeled the ε band of the hemochromogen and is readily observed in hemoglobin, myoglobin, and cytochrome c. Drabkin has previously suggested that this band is due in part to heme absorption (15). In these proteins, however, the band could not be delineated from the absorption bands of the aromatic chromophores. Indeed, the 277 μ peak in the hemoepoiesis is studded with vibrational components from the aromatic amino acid absorptions.

A second significant feature can be distinguished from the absorption curves of the heme peptide. It is possible to assess the contribution of the heme to the absorption at 190 μ. In the peptide portion there are 10 peptide chromophores and 2 amides, in addition there are 2 ionized carboxyl groups on the peptide and 2 on the heme moiety. Since the absorption of the 190 μ band of polypeptides is dependent on the conformation of the protein (and on any dispersion force interactions with the heme transitions), both the helical and random coil absorptions should be considered. The millimolar extinction coefficient for the random coil is approximately 7 at 190 μ (16, 17). The millimolar extinction coefficient for an ionized carboxyl group may be taken at 1.6 (18). Including the amide chromophores with the peptide chromophores and adding the molar absorption of the carboxylate groups, a millimolar extinction coefficient of 90 is obtained for the peptide and carboxylate groups. The value for the heme peptide, which has some dependence on oxidative state, is approximately 120. Thus, the millimolar extinction coefficient at 190 μ for the heme, imidazole, and thiol ether bridges would be about 30. If the peptide had extinction coefficients more nearly that of helical protein (ε_{280} = 42), then the absorption due to heme, imidazole, and thiol ether groups would account for about 50% of the absorption, i.e. a millimolar extinction coefficient of 60 at 190 μ. On the basis of the amino acid absorptions (19) the two thiol ether groups and the imidazole moiety are not expected to account for more than a combined ε_{280} of about 8. Thus, it is apparent that the heme contributes substantially to the absorption in the peptide region. In the following it will be perhaps more obvious that the contributions to circular birefringence and circular dichroism in the peptide range are even more striking.

Optical Rotation of Heme Peptide—There are many features of
Interest in the ORD curves of the heme undecapeptide, some of which have been discussed previously (20). For completeness, the entire curves from 600 μm to 190 μm are given in Fig. 2. The complex Soret Cotton effects which result from aggregation have been treated in detail elsewhere (21). At shorter wavelengths there is much optical activity which is dependent on the oxidative state of the heme. The details of these rotations are most readily discussed with reference to the circular dichroism data in Figs. 3 and 4. As is clearly observed in the Soret region the optical activity of the hemec is dependent on aggregation (21). For this reason those systems will first be considered which have been shown to be monomeric. A nearly Gaussian Soret band is indicative of the monomer (21). Addition of 0.25 M imidazole at pH 9 produces the monomer and allows study to about 240 μm. The monomer may also be obtained by dilution and by raising the temperature, but the long path lengths required prevent study below 205 μm. It may also be noted that obtaining monomer by dilution and elevated temperatures (50°) does not allow reduction by dithionite. At a concentration of 3 M and 50°, addition of dithionite effects a reduction followed by a time-dependent loss of the Soret band and the appearance of a large positive CD band at 315 μm. It should be noted that

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**Fig. 1.** Absorption curve of ferroheme undecapeptide (-----) and ferriheme undecapeptide (- - -) in 0.05 M sodium tetraborate at pH 9. The right hand ordinate is for the Soret band and the short wavelength absorption from 210 μm to 190 μm. Thus the α, β, δ, and ε bands should be read on the left hand ordinate scale. The value of ε₃₅₀ is close to 120 at 190 μm for both the ferro and ferri forms. An ε hemochromogen band is found at 277 μm.

**Fig. 2.** Optical rotatory dispersion curve of ferroheme undecapeptide (-----) and ferriheme undecapeptide (- - -). The left ordinate is for sections a (190 μm to 260 μm) and c (380 μm to 460 μm). The right ordinate is for sections b (260 μm to 380 μm) and d (460 μm to 600 μm). The heme-peptide concentration for sections a and c was 1.57 mM and for sections b and d 4.45 mM. The buffer was 0.05 M sodium tetraborate at pH 9 and the temperature was 27°. Rotation is reported on a molar heme basis.
optically active heme transitions in the 300 nm to 240 nm range. The 278 nm CD band corresponds to the 277 nm ε hemochromogen band. The 283 nm CD extremum has no readily observable counterpart in the hemochromogen spectrum. Similarly, the 253 nm band of ferriheme has no absorption counterpart. These transitions may be electrically forbidden but magnetically allowed heme transitions. The dependence of the bands on oxidative state re-emphasizes that they are heme transitions. These results are in direct contradiction to previous conclusions that cytochrome heme transitions do not contribute to optical rotation in the region of the aromatic side chain chromophores (20). The CD data for the heme undecapeptide in the presence of imidazole (Fig. 3) is remarkably similar to the CD of ferri- and ferrocytochrome C from Azotobacter vinelandii.2

The CD curves of the heme undecapeptide may be determined at shorter wavelengths under conditions in which aggregation occurs. Fig. 4 shows the CD data for a 0.3 mM heme peptide solution. The most striking feature of this curve is the dependence of the ellipticity on the oxidative state of the heme. On reduction there is an increased negative circular dichroism between 205 nm and 260 nm. This change must be ascribed, at least in part, to optically active transitions within the heme or the coordinated imidazole. It cannot be assumed that the optical rotation of the heme peptide at short wavelengths is dominated by the so-called intrinsic Cotton effects of the peptide amide bonds (20). The ferriheme undecapeptide exhibits a negative peak at 202 nm and a weak shoulder just short of 220 nm. Whereas the 220 nm shoulder is suggestive of the 222 nm negative dichroic peak of the α-helix, the strong negative peak at 202 nm is more suggestive of the random coil. The ellipticity on a mean residue basis of the 202 nm peak is approximately $1 \times 10^4$, which is low for disordered chains. The mean residue ellipticity at 222 nm is approximately $0.3 \times 10^4$. As the value for the 222 nm extremum of helical proteins is 10 times greater, a calculation of helical content would result in a value of 10%. Since only 10 peptide chromophores are involved, a value of 10% is

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**Fig. 3.** Circular dichroism curves for monomeric heme undecapeptide. Imidazole at a concentration of 0.25 M was used to obtain monomer in the pair of curves for oxidized (---) and reduced (-----) heme peptide. Dilution (3 μM) and elevated temperatures (50°C) allowed determination of the ferriheme undecapeptide (-----) in the absence of added ligand. The ellipticities (θ) are on molar heme basis.

**Fig. 4.** Circular dichroism curves of 0.3 mM heme undecapeptide for the reduced (-----) and oxidized (---) states in the 190 nm to 370 nm wavelength interval. Note the increased negative dichroism in the 205 nm to 260 nm range of the reduced form. The ellipticities (θ) are on a per mole heme basis.
FIG. 5. Circular dichroism curves of oxidized (-----) and reduced (——) horse heart cytochrome c. These curves are to be compared to those in Figs. 3 and 4. The ellipticities (θ) are on a molar heme basis.

would mean that only one residue is in helical array. Of course, such a statement has no meaning. The CD data indicates that the peptide moiety has no regular geometrical relationship between the peptide chromophores in the ferriheme undecapeptide, that is, the data most closely resemble those of a random coil. It is also apparent from comparison of Figs. 3 and 4 that there is some dependence on aggregation.

In the discussion to this point reference has been made to extrema observed in the absorption and circular dichroism spectra. The combination of these data afford the opportunity to attempt resolution of complex curves into a set of Gaussian functions which simultaneously fit both the rotation and absorption data. Although it is not possible to claim a unique resolution into simple functions, the resolved curves do present the data in a manner which allows improved quantitative comparison of this heme chromophore data with that of the parent molecule, cytochrome c, and other heme proteins. These comparative studies are now in progress. The procedure, with the use of the visual DuPont 310 curve resolver, is to first approximate the circular dichroism curve with a minimum of Gaussian functions. Without varying width or position of these Gaussian functions, an attempt is made to fit the absorption curve by displaying the functions in a positive mode and changing only the height of the curves. The functions are then altered until an apparent minimum, common set simultaneously fit both curves by changing only amplitude and sign.

The resolved Gaussian functions for the ferriheme undecapeptide data in 0.05 M sodium tetraborate are given in Table I. The resolution into four bands is quite straightforward, three bands being apparent in the complex curves. The resolved band at 249 μm has a relatively large anisotropy, indicative of a large magnetic moment for the transition. The bands at 329 μm and 354 μm can be seen in the absorption as a shoulder and peak, respectively, and form the broad negative CD band (Fig. 4). The 288 μm band is only slightly apparent in absorption. The resolved Gaussian functions for the ferroheme undecapeptide are more in number (Table II). The most prominent feature is a band at 262 μm. This band is the result of an electronic transition with a large magnetic moment. It is expected that the anisotropy is a minimal value as a consequence of the procedure of curve resolution. The 262 μm band is of further interest because a similarly placed band is found on ligand binding to myoglobin and hemoglobin (Figs. 6 and 7). Specifically, oxygen, carbon monoxide, cyanide, and azide derivatives of myoglobin all result in an intense CD band at about 260 μm with relatively small changes in absorption at this wavelength. This band is presently being correlated with spin state. A resolved band with substantial dipole strength as well as rotational strength is found at 281 μm. The bands at 262 μm and 281 μm serve to emphasize the contribution of the heme chromophore to the absorption and optical rotation in the so-called “aromatic” region.

Circular Dichroism of Heme Proteins—It is necessary to discuss the ultraviolet CD curves of cytochrome c, myoglobin, and hemoglobin in order to note the dependence of optical rotation of these heme proteins on the bound ligand and on the oxidative state.
TABLE I

Critical values for resolved Gaussian curves

<table>
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<tr>
<th>Wavelength of</th>
<th>Molar extinction</th>
<th>Dipole strength$^a$</th>
<th>Molar ellipticity</th>
<th>Rotational strength$^b$</th>
<th>Anisotropy $(R_i/D_i)_{10}$</th>
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$^a$ The dipole strength is calculated from the expression (23)

$$D_i = 1.23 \times 10^{-20} \frac{\epsilon_i \Delta \lambda_i}{\lambda_i}$$

where $\epsilon_i$ is the molar extinction coefficient at the curve maximum, $\lambda_i$ is the wavelength of the $i^{th}$ maximum and $\Delta \lambda_i$ is the half band width at $\epsilon_i/e$.

$^b$ The rotational strength is calculated from the expression (23)

$$R_i = 1.65 \times 10^{-42} \frac{\theta_i \Delta \lambda_i}{\lambda_i}$$

where $\theta_i$ is the molar ellipticity at the curve maximum $\Delta \lambda_i$ and $\lambda_i$ are as defined above.

$^c$ The significance of anisotropy is discussed by Kauzmann, Walter, and Eyring (24).

and to compare the changes with those observed in the native peptide. Future work will treat these systems more exhaustively in an attempt to determine the contributions of the aromatic residues. $^3$ The data on the heme undecapeptide of cytochrome c are most closely related to those of the parent molecule (Fig. 5). Reduction of cytochrome c results in a negative displacement of the CD curve in the 200 $\mu m$ to 250 $\mu m$ range. A similar negative displacement was observed for the heme peptide in which the differences between native ferri- and ferrocyanochrome c, which have been observed in the ORD (1–4) and which are now seen in the CD, would seem most directly to be accounted for in terms of optical activity of the heme moiety and not reflect a change in helical content. The possibility that this negative displacement may also be due to a change in the optical activity of the coordinated imidazole is also being investigated. This does not alter the conclusion of helix formation on reduction of ferri-cytochrome c in guanidine hydrochloride (1, 3). There continues to be a qualitative correspondence between the heme peptide data and those of cytochrome c in the aromatic region. The curve of the reduced heme protein has a positive extremum at 264 $\mu m$ with an amplitude of nearly twice that of the corresponding peak in the heme undecapeptide. The positive 278 $\mu m$ band of the ferroheme undecapeptide is not observed in the parent molecule. Oxidized cytochrome c has a positive band of 288 $\mu m$.

**TABLE II**

Critical values for resolved Gaussian curves

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<th>Wavelength of</th>
<th>Molar extinction</th>
<th>Dipole strength$^a$</th>
<th>Molar ellipticity</th>
<th>Rotational strength$^b$</th>
<th>Anisotropy $(R_i/D_i)_{10}$</th>
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$^a$ The dipole strength is calculated from the expression (23)

$$D_i = 1.23 \times 10^{-20} \frac{\epsilon_i \Delta \lambda_i}{\lambda_i}$$

where $\epsilon_i$ is the molar extinction coefficient at the curve maximum, $\lambda_i$ is the wavelength of the $i^{th}$ maximum and $\Delta \lambda_i$ is the half band width at $\epsilon_i/e$.

$^b$ The rotational strength is calculated from the expression (23)

$$R_i = 1.65 \times 10^{-42} \frac{\theta_i \Delta \lambda_i}{\lambda_i}$$

where $\theta_i$ is the molar ellipticity at the curve maximum $\Delta \lambda_i$ and $\lambda_i$ are as previously defined.

$^c$ The significance of anisotropy is discussed by Kauzmann, Walter, and Eyring (24).

$^3$ D. W. Urry, to be presented at the Symposium on Cytochromes, Osaka, Japan, August 16–18, 1967.

**FIG. 6.** Circular dichroism curves of horse heart myoglobin in the oxidized (——) and reduced (· · · ·) forms and as the carbonyl (-----) and oxygenated (——) derivatives. Ellipticities ($\theta$) are on a molar heme basis.
approximately the same magnitude and location as in the ferrihemeproteins but it is more complex. The one feature which clearly distinguishes cytochrome c from the heme peptide is the fine structure. This distinction is also observed in myoglobin and hemoglobin and may provide a means for delineating the contribution due to the aromatic residues. These localized peaks correspond to slight peaks on the absorption curves of the heme proteins and in turn, may be correlated with vibrational fine structure of the aromatic amino acids (19).

The curves for myoglobin (Fig. 6) exhibit positive CD bands of larger magnitude in the 250 µm to 270 µm wavelength interval. Again, these curves are studded with vibrational fine structure. If information on possible changes in the environment of given aromatic residues is to be obtained from CD data, it would seem that the vibronic bands must be resolved and that the sources of rotational strength of such bands must be understood. Work toward that end is in progress in this laboratory. Although the data are not included in Fig. 6, it may be noted that the difference between oxy- and deoxymyoglobin in the peptide region is less than in hemoglobin (see Fig. 7).

The CD data on hemoglobin also exhibit differences between the deoxy- and oxy-forms which are suggestive of the differences between ferri and ferrohemeproteins. It is significant in this regard that deoxyhemoglobin and the ferriheme undecapeptide have parahematin type spectra, whereas oxyhemoglobin and reduced heme undecapeptide display hemochromogen type spectra. In the peptide region, oxyhemoglobin, rather than exhibiting an increased negative dichroism with respect to deoxyhemoglobin, is found to be more positive in accord with the ORD data of Frankel and Doty (24) and Brunori et al. (25). The change is the reverse of what is found in the model hemepeptide. This distinction may prove important when the CD data of myoglobin and hemoglobin are compared in detail. For example, this difference could reflect a changing heme-imidazole orientation in hemoglobin which could, in effect, trigger the conformational changes giving rise to facilitated oxygenation.

The heme proteins—cytochrome c, myoglobin, and hemoglobin—all exhibit circular dichroism bands in the 300 µm to 240 µm range in which the ratio of rotation to absorption is greater than that of the Soret band. The bands near 260 µm are of the same sign in the heme proteins as similarly positioned bands in the heme undecapeptide. The ellipticities are the same or greater in the proteins. The larger values may be due to interactions with the protein which result in larger rotational strengths, they may in part be due to the contribution of other groups in the protein, or most reasonably, they are due to bound ligands and may be correlated with the spin state. What is sets the heme proteins apart from the hemepeptide are the localized rotational bands which correspond to the vibrational fine structure in the absorptions of the aromatic amino acids. In myoglobin and cytochrome c, the bands corresponding to wavelengths of vibrational fine structure in the 270 to 290 µm range appear to be negative and riding on positive backgrounds. The interesting feature is that bands are not readily resolved which follow the general absorption of the amino acids in this region. If the negative 289 µm band in cytochrome c is due to interaction

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4 R. Frankel and P. Doty, personal communication.
of tryptophan with heme (26), then its presence in hemoglobin and myoglobin may allow an interesting correlation. The tryptophan near the heme in horse hemoglobin is residue C3 (27). However, the narrow bands near 290 m\mu have been observed in carbonic anhydrase (28–31). Their presence is not exclusive to heme proteins and therefore to heme-aromatic residue interactions. The CD changes observed in the 200 m\mu to 240 m\mu range are provoking because of the possible sources and implications, but substantive interpretation will be difficult and will require careful work on model systems in which alternative explanations can be eliminated.

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