Model Systems for Interacting Heme Moieties. I. The Heme Undecapeptide of Cytochrome c[1]

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Abstract: The monomeric and polymeric states of the heme undecapeptide were examined using optical rotatory dispersion, circular dichroism, and difference absorbance measurements. In terms of difference absorbance the aggregation is seen as a hyperchromism and a long wavelength shift of the Soret band. The hyperchromism data are taken to indicate that there are dispersion force interactions between the heme moieties which suggest a more nearly head-to-tail alignment of heme planes than stacking in a card pack fashion. The shift to longer wavelengths of the Soret absorption band as a result of aggregation is in accord with exciton resonance interactions which also suggest a head-to-tail alignment. In terms of optical rotatory dispersion the aggregated state is characterized by a complex Soret-Cotton effect. In circular dichroism the multiple extrema are seen to be of alternating sign. The optical rotation data show in greater detail the exciton splitting of the Soret band. This is most emphatically observed in the ferroheme undecapeptide where a minimum of three discrete electronic transitions are seen within the confines of the Soret band. The aggregated heme peptides are presented as model systems for studying the interaction of heme moieties with the view of establishing criteria for determining the proximity of hemes in more complex biological systems, for example, in active enzyme systems from the electron-transport chain. The results are discussed with respect to the possible juxtaposition of heme moieties in ferrocytochrome oxidase and with respect to detecting heme–heme interaction potentials in hemoglobin.

In the cytochrome region of the electron-transport chain, electrons are transferred from the heme of one cytochrome to that of a subsequent cytochrome in the sequence. It would be of considerable interest to know if the heme moieties are juxtaposed or if they pass within a distance of less than 10–15 A during the transfer process. Specifically, in the case of cytochrome oxidase, which contains two hemes, knowledge of the relative orientation of these hemes is fundamental to understanding the mechanism of electron transfer. Before the question of heme proximity can be adequately resolved, it is necessary to establish, with model systems, the characteristics of interacting heme moieties and to confirm or develop a basis with which to interpret the biological system.

The intense Soret band, with molar extinction coefficients greater than 100,000, gives rise to interaction potentials between transition dipole moments which may exist over large distances. Should heme moieties aggregate with heme–heme distances of less than 10–15 A, an interaction potential between transition dipole moments may be expected which would lead to exciton splitting of the Soret band energy. Such splitting, where detectable, could be used to determine the structure of the polymer.[2] As is demonstrated in the present communication, optical rotatory dispersion (ORD) and circular dichroism (CD) are sensitive probes for detecting exciton splitting in optically active heme systems. Absorption spectroscopy is another optical method which may be used to detect exciton resonance interactions and dispersion force interactions. The latter also lends itself to structural characterization of a polymer.[3-5] Detailed studies of optical rotation and absorption changes attending aggregation of optically active heme polymers allow calibration of these methods for detecting the presence or absence of interacting hemes in more complex heme protein systems.

Peptic digestion of cytochrome c yields a small heme peptide in which the heme is bound to an 11 amino acid peptide of known sequence.[6,7] Attachment is by two thioether linkages to the α carbons of the saturated vinyl groups on two pyrrole moieties.[8-10] This heme undecapeptide provides an optically active system which is known to aggregate.[10] Furthermore, in the polymerized state it is to be expected that the hemes are sufficiently close to give rise to detectable interaction potentials. Indeed aggregation-dependent hyper- and hypochromism has been observed for heme peptides.[11] In order to properly assess the effects of interactions within the aggregates, the properties of the monomer must be clearly determined and compared to those of the polymer. To do this we shall use as a basis the ultracentrifugation data of Ehrenberg and Theorell.[10] Their sedimentation studies indicate that the heme undecapeptide is monomeric and monodisperse in citrate at acid pH, that it is a monodisperse polymer in borax at alkaline pH, and that addition of histidine results in depolymerization of the aggregate. The heme undecapeptide system, therefore, provides a model in which to study the interaction of heme moieties and thereby leads to a means of determining proximity and relative orientation of heme groups. The spectroscopic tools sensitive to regular arrays of identical groups may be experimentally calibrated for heme systems by using the heme peptide aggregates. In this communication we treat the heme undecapeptide.

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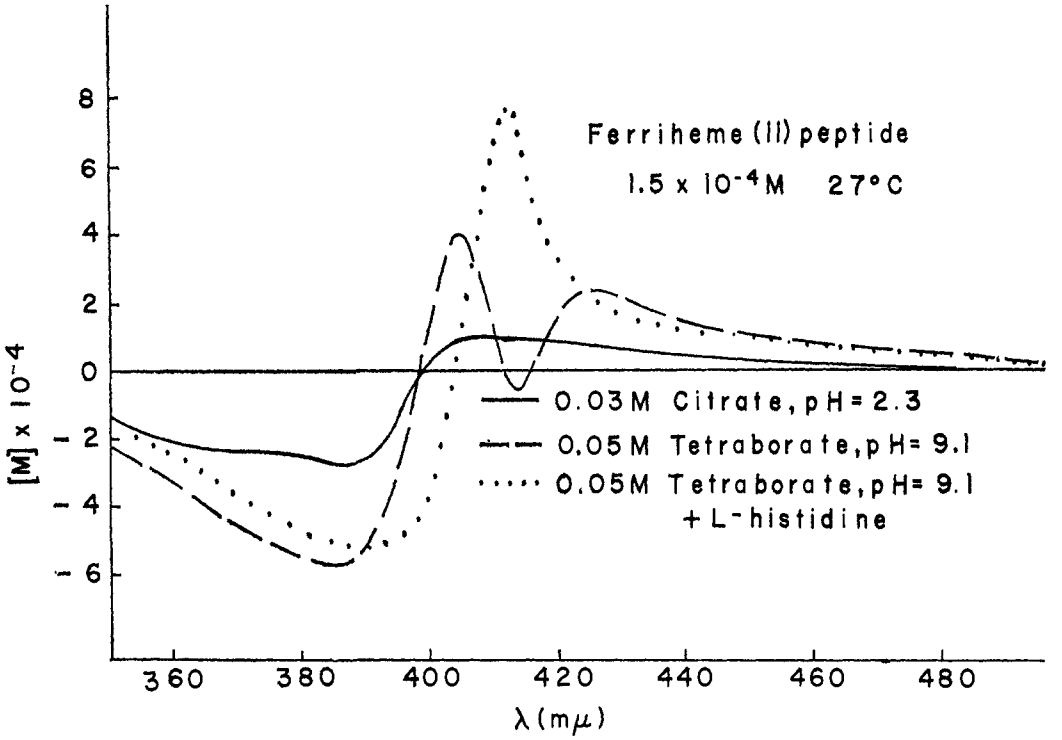

Figure 1. Optical rotatory dispersion in the Soret region of ferriheme undecapeptide in the monomeric state in citrate, ——, and tetraborate + histidine, · · · · ·, and in the polymeric state in tetraborate, - - - - -. The Cotton effect is complex in the aggregated state.

The second paper in this series concerns the heme octapeptide which exhibits very different circular dichroism curves and a hypo- rather than a hyperchromism.

While emphasis in this communication is on the polymer it should be noted that careful characterization of the monomer provides information on the sources of the rotational strength of the Soret band. This insight, gained by studying the simpler heme peptides, will allow more detailed interpretation of the Soret-Cotton effects as related to the heme environment in heme proteins in general, and in cytochrome *c* in particular. Knowledge of the heme environment is essential in elucidating the mechanism of oxidation and reduction of cytochromes as well as in determining the mechanism of oxygenation of myoglobin and hemoglobin. In the latter regard it may be noted that the properties of the aggregate are relevant to the so-called "heme–heme interaction" in hemoglobin. Should a substantial interaction potential exist between the hemes of hemoglobin then one would expect the Soret circular dichroism curve to be complex rather than to approximate a simple Gaussian function. The dipole interaction potential for exciton splitting has an R^{-3} distance dependence whereas the distance dependence for van der Waals interaction is R^{-6}. From considerations of distance dependence and the magnitude of the Soret transition dipole moment, one might expect a splitting of the Soret band energy if "heme–heme interaction" were due to polarizability interactions between hemes.

Experimental Section

The heme undecapeptide was prepared as outlined by Harbury and Loach.[12] The amino acid composition of the peptic digestion product of horse heart cytochrome *c* agreed very well with the reported amino acid sequence of the heme peptide.[7,13] Cytochrome *c* (type III), citrate, histidine, and imidazole were obtained from Sigma Chemical Co. The reduced form was obtained by addition of a few grains of dithionite. Determinations of pH were made on a Radiometer pH meter, Model 25SE. Sample temperatures were maintained with the Haake KT-62 Kryothermat and were monitored with a YSI Model 42SC Telethermometer while spectra were being run. Cell path lengths were calibrated using solutions of chromate in 0.05 *N* KOH. Absorption and optical rotatory dispersion curves were determined on a Cary Model 14 spectrophotometer and a Cary Model 60 spectropolarimeter, respectively. Circular dichroism curves were run on a prototype unit built by Cary Instruments for the Model 60. The prototype CD unit was calibrated using the Cary Model 1401 circular dichroism attachment for the Model 14. The standard used was an aqueous solution of *d*-10-camphorsulfonic acid (J. T. Baker lot no. 9-361) with an $\epsilon_L - \epsilon_R$ of 2.20 at 290 mμ.

Results

Our approach in the characterization of the heme undecapeptide is to use the sedimentation studies of Ehrenberg and Theorell[10] as a basis for the empirical correlation of changes observed in the optical rotation data with changes in the degree of polymerization. The sedimentation data for heme peptide in 0.03 *M* citrate at pH 2.3 indicated a monomeric and monodisperse solute with a molecular weight of 2000. The solid curve in Figure 1 is the optical rotatory dispersion curve for the same system. The curve exhibits a simple Soret–Cotton effect with some distortion on the negative lobe which is due to the shoulder on the short wavelength side of the Soret band. At pH 9 in 0.05 *M* sodium tetraborate, conditions under which the polymeric form exists, a complex Soret–Cotton effect is observed with an additional negative extremum at 413 mμ (see Figure 1, dashed curve). Depolymerization of the alkaline aggregate was reported to occur in

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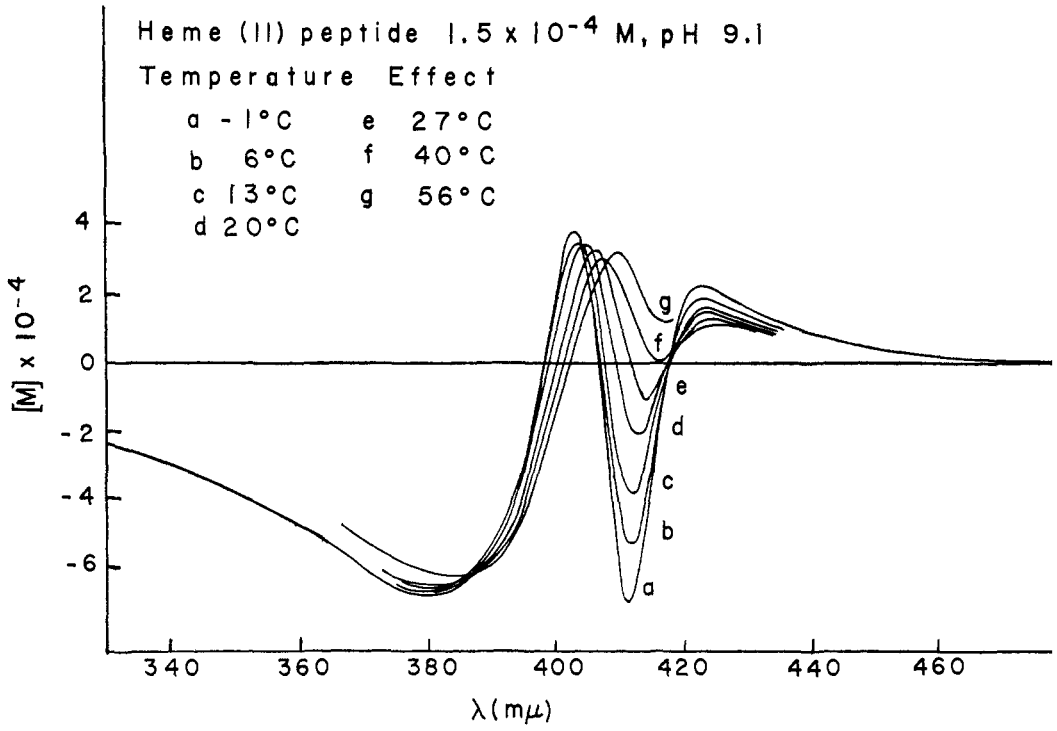

Figure 2. Temperature study of the aggregation of ferriheme undecapeptide followed by optical rotatory dispersion. The complexity of the Cotton effect is dependent on temperature. The complexity decreases with increasing temperature as is to be expected on disaggregation.

the presence of histidine. It is apparent, by comparing the curves in Figure 1, that the presence of histidine causes a loss of the complexity observed in the dashed curve and results in the simpler Cotton effect seen in the dotted curve. Thus it would appear that a relatively simple Cotton effect is diagnostic of the monomeric form and the presence of a negative peak in the ORD at 413 mμ is indicative of the presence of polymeric forms.

In order to substantiate the preceding empirical correlation, temperature and concentration studies were pursued. Increases in temperature and decreases in concentration should favor the monomeric species and result in a decreased complexity of the ORD curve. The temperature effect over the range −1 to 56° is reported in Figure 2 where an exceedingly regular loss of complexity is found as the temperature is raised. The incremental increase in magnitude of the negative peak for a given temperature interval is just as large from −1 to 6° as from 6 to 13°, indicating that there is a continued increase in aggregation at the lowest temperature reached. Band sharpening at lower temperatures may also add to the increased amplitude of the peaks. The effect of varying the concentration over a 1000-fold range is seen in Figure 3. The temperature of the concentration study was 34°. This is the lowest temperature at which the most dilute sample (1.5×10^{-6} *M*) showed no negative peak in the vicinity of 413 mμ. There is a regular loss of complexity and therefore a regular decrease in the degree of aggregation as the sample is diluted. The simple Cotton effect obtained at a concentration of 1.5×10^{-6} *M* and a temperature of 34° serves to define the monomeric species without resorting to low pH or to the addition of ligands such as histidine or lysine.

The definition of a monomer, achieved by dilution and by slightly raising the temperature, allows determination of the hyperchromism attending aggregation without the added complication of ligand binding effects which may alter the extinction coefficient. The hyperchromism is best observed as a difference absorbance in which the monomer solution, defined by the ORD studies, is placed in the reference beam, and solutions of varying degrees of polymerization are positioned in the sample beam. The path length is varied reciprocally with the concentration such that the same number of subunits are in each beam. The resulting curves are given in Figure 4. Clearly, there is an increase in absorption on polymerization. The oscillator strength per subunit increases by 15%. The vertical line crossing the zero line marks the position of the monomer Soret maximum. The maximum in the difference absorbance is shifted to the red of the monomer peak. Thus, associated with the hyperchromism, there is a long wavelength shift in the band center.

A more direct examination of the energy splitting of the Soret band is possible with circular dichroism. Absorption and CD curves of the ferriheme undecapeptide are presented in Figure 5. In the presence of 0.25 *M* imidazole the CD curve is very nearly Gaussian in shape and peaks at 402 mμ. The principle distortion is a more gradual slope on the short wavelength side of the CD curve which corresponds to a similar distortion on the short wavelength side of the Soret band. At a concentration of 3×10^{-4} *M* the CD curve exhibits two positive extrema at 397 and 417 mμ and a negative extremum at 408 mμ. A minimum of two Gaussian curves would be required to fit the observed data, that is, a narrow negative band at about 408 mμ and a broader positive band at slightly shorter wavelengths. Proper fitting of the curves requires more Gaussians (see Discussion). Thus the Soret band of ferriheme undecapeptide contains at least two discrete

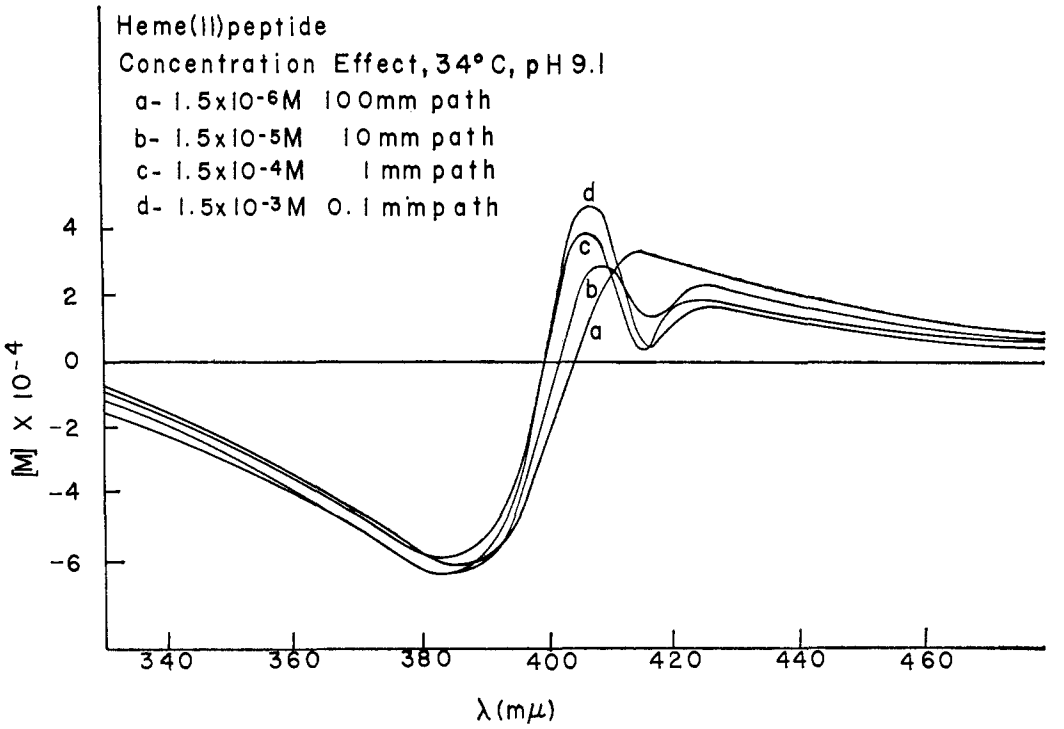

Figure 3. Concentration effect at 34° of the ferriheme undecapeptide. With dilution the complexity of the ORD curve decreases as is to be expected on disaggregation. The most dilute solution, 1.5×10^{-6} *M* at 34°, serves to define the monomeric state in the absence of added ligands and without going to low pH.

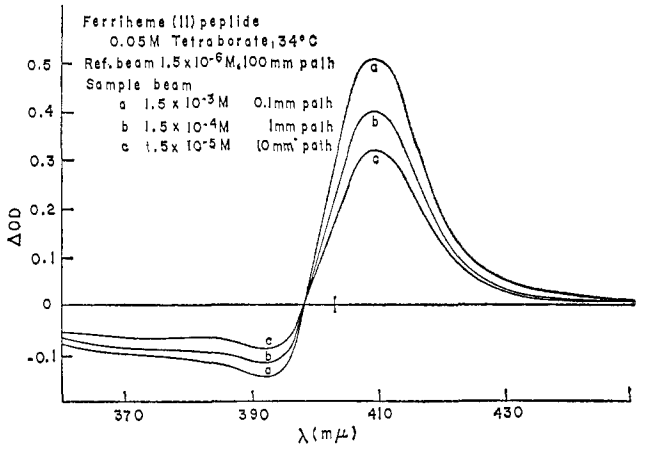

Figure 4. Difference absorbance studies in the Soret region with the monomer, defined in the figure in the reference beam and with increasing concentrations and reciprocally decreasing path lengths in the sample beam. A hyperchromism and shift to longer wavelengths is observed in the Soret band as concentration is increased.

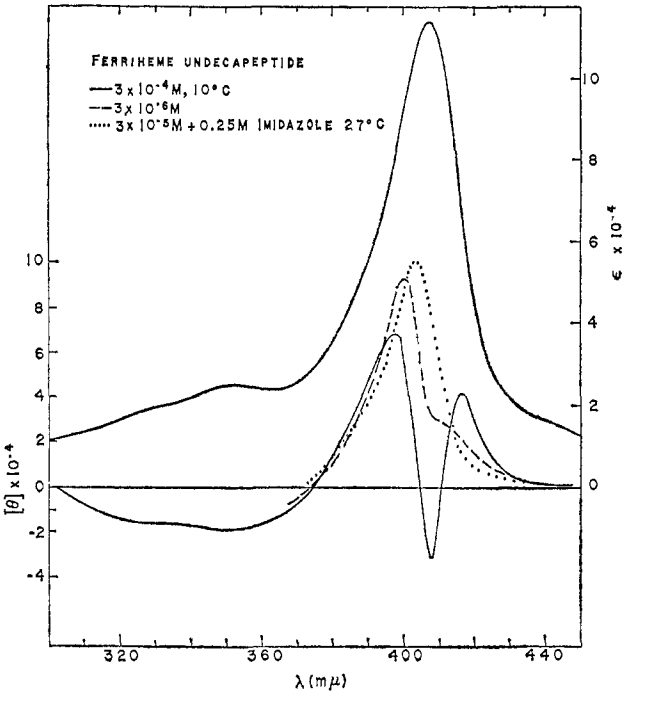

Figure 5. Absorption curve and circular dichroism curves of the Soret band of ferriheme undecapeptide. The effect of concentration and of added ligand is seen in the complexity of the CD band. The presence of at least two discrete transition energies are seen within the envelope of the Soret absorption band.

transition energies. Also included in Figure 5 is a second concentration to show the effect of dilution on the complexity of the band. Circular dichroism and absorption curves for the reduced undecapeptide are given in Figure 6. Addition of 0.25 *M* imidazole to 3×10^{-5} *M* ferroheme undecapeptide results in a relatively simple Soret CD band with a positive extremum at 415 mμ. At concentrations of 3×10^{-4} *M* a CD curve with multiple extrema is found. The Cotton effect decreases slightly in amplitude but retains the complexity at 3×10^{-6} *M*. The Soret band of ferroheme undecapeptide exhibits four CD extrema of alternating sign. Positive peaks occur at 425 and 416 mμ, and the negative peaks are at 420 and 405 mμ. It is significant to emphasize that a minimum of three Gaussian functions would be required to approximate these data and that adequate fitting of the curve seems to require four functions. As all these extrema fall within the width of the Soret band (see Figure 6) it is apparent that the Soret band contains at least three discrete energies.

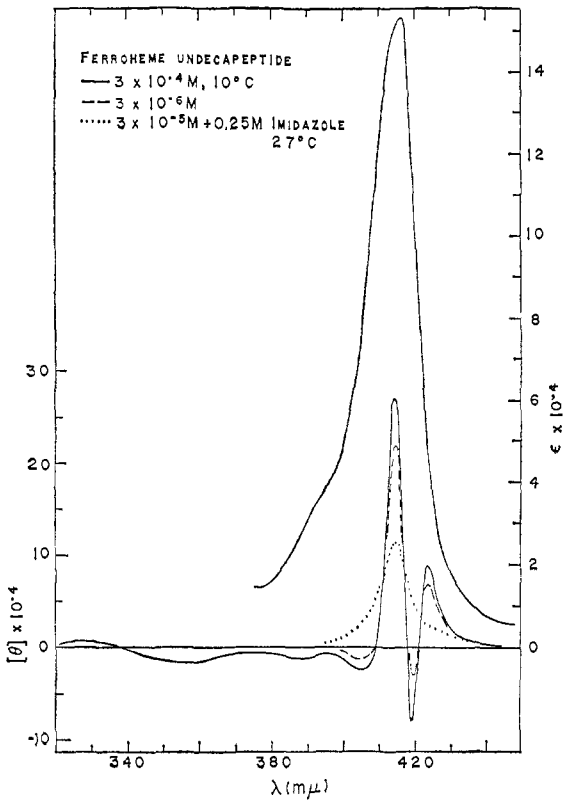

Figure 6. Absorption curve and circular dichroism curves of the Soret band of ferroheme undecapeptide. The effects of concentration and added imidazole are seen in the complexity of the CD band. The presence of at least three discrete transition energies is seen within the envelope of the Soret band. This emphatically demonstrates the utility of CD to detect the splitting of energies within a single absorption band.

The ability of circular dichroism to resolve these energies when the absorption curve gives no suggestion of such detail most emphatically demonstrates the utility of this technique to detect exciton resonance interactions.

Discussion

A splitting of the Soret band energy has been observed which is dependent on the aggregated state. Associated with this energy splitting and, of course, dependent on the presence of polymer is a hyperchromism and a bathochromic shift of the band. These effects may be discussed in terms of exciton splitting of the Soret transition energy and in terms of dispersion force interactions. Considerations of dispersion force interactions and of exciton splitting may be qualitatively applied to the ferriheme undecapeptide aggregate to determine the relative orientation of heme planes. The optical rotation and absorption data should provide a consistent picture.

Expressions relating hyper- and hypochromism to geometry in polymeric systems have been derived by Tinoco[3] and by Rhodes.[4] If the transition under study is in the plane of an aromatic chromophore and if one takes the polarizabilities in that plane to be greater than the polarizability perpendicular to the plane, then hyper- or hypochromism of the band under study provides information on the relative orientation of the groups within the polymer which comprise the chromophore. Specifically, if the planar aromatic groups are stacked in a card pack fashion, then there is a decrease in the area of the absorption band on a per absorbing unit basis when compared to the randomly oriented aromatic groups. Should the chromophoric groups be oriented head to tail, then there would be an increase in the area of the absorption band on a per absorbing unit basis when compared to the disoriented case. Thus the former case, hypochromism, or the latter case, hyperchromism, may be used in this qualitative manner to determine the relative orientation of planar aromatic systems.

An aggregation-dependent splitting and/or shifting of a band under study may also be used to gain qualitative information on the relative orientation of the transition dipole moments responsible for the band. When the transition dipole moment has been fixed with respect to the molecular framework, one then has information on the relative orientation of the chromophoric groups. An excellent discussion of this aspect is due to Kasha.[2] When considering exciton interactions, several useful limiting cases are commonly treated (see Figure 4 of ref 2). A parallel stacking of transition dipoles leads to a blue or hypsochromic shift in the absorption band position; a head-to-tail alignment of transition dipoles effects a red or bathochromic shift in the energy of the transition; and an oblique orientation results in band splitting about the wavelength of the monomer transition. Thus if the aggregation of a planar, aromatic group with a large transition dipole moment is under study, then one might expect hyperchromism to be associated with a bathochromic shift or hypochromism to be associated with a hypsochromic shift of the band in question. Such a consistent picture may be obscured, for example, by solvent effects or by the inadequacy of the dipole–dipole interaction potential approximation.

As the polarizabilities in the directions of the heme plane are much greater than the polarizability perpendicular to that plane, the dispersion force interactions giving rise to hyperchromism in the heme undecapeptide system may be used to provide information on the relative orientation of heme planes. Thus the hyperchromism suggests that the hemes in the aggregate are in a more nearly head-to-tail alignment than in card pack fashion. The Soret band is a doubly degenerate transition with the transition dipole moments in the plane of the ring.[14,15] Accordingly, general band shifts due to exciton interactions also give information on the relative orientation of heme planes. The bathochromic band shift seen in difference absorbance (Figure 4) as a function of concentration is to be expected for an aggregate in which the alignment of hemes is more nearly head to tail than stacked. The data appear consistent.

Iron binding ligands such as lysine, imidazole, histidine, and ammonia disperse the ferriheme undecapeptide aggregate.[11,16] In view of the ligand effects and the known amino acid sequence of the heme peptide, it is likely that the ϵ-amino group of lysine is the group effecting the polymerization. This likelihood is fur-

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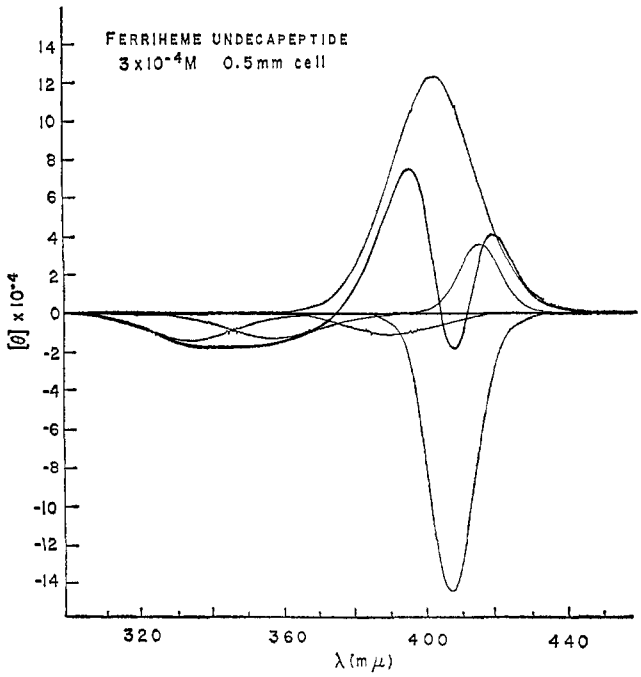

Figure 7. Resolution of the CD curve reported in Figure 5 into a set of Gaussian curves which may also be used to describe the corresponding absorption curve (Figures 5 and 8).

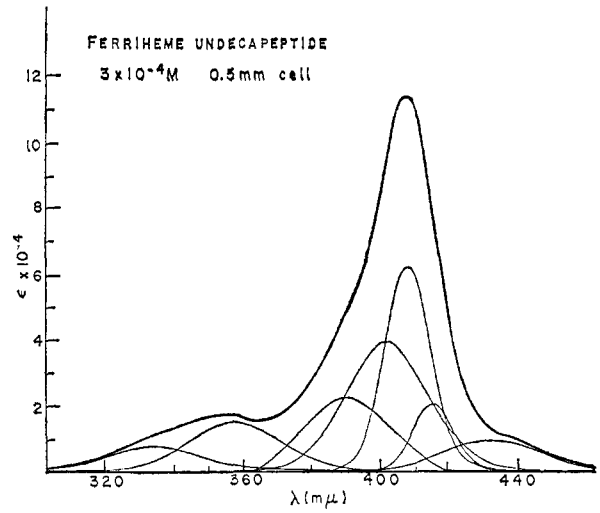

Figure 8. Resolution of the absorption curve reported in Figure 5 into a set of Gaussians which by varying height and sign will simultaneously describe the CD curve (Figure 7).

ther supported by the aggregation properties of the heme octapeptide in which the lysine residue has been removed. The heme octapeptide exhibits a marked hypochromism on aggregation rather than hyperchromism and the optical rotatory dispersion[17] and circular dichroism[18] curves are of a different form than those of the heme undecapeptide. Taking the ε-amino group of lysine to be the polymerizing unit, an approximation of the maximal distances between hemes can be obtained from molecular models. Using the Corey–Pauling–Koltun models the maximum distance between heme centers is about 16 A. The distance between the closest pyrroles is about 11 A. If we construct a reasonably compact structure with the hemes in a primarily head-to-tail fashion but with a displacement from coplanarity, the distance between heme centers would be 9–10 A. With these structural limitations and considerations in mind we may now consider the resolution of the CD and absorption curves into Gaussian curves in an attempt to determine the splitting energies. Assuming a dipole interaction potential, the splitting energies can determine a maximal distance between hemes.

The problem of resolving a given curve into a sum of Gaussian curves lies in determination of the uniqueness of a given solution. Our approach, using the visual Du Pont 310 curve resolver, was first to approximate the circular dichroism curve with a minimum of Gaussian functions. Then, without varying the width or position of the Gaussians, an attempt was made to fit the absorption curve by displaying the functions in a positive mode and by changing only the height of the curves. Gross misfitting was corrected for by addition of a new Gaussian or by slightly varying the width and position of existing curves. The process of shifting between CD and absorption was continued until a common set of bands could approximate both CD and absorption curves by changing only sign and amplitude. There is no attempt to argue that the resultant set of curves (Figures 7 and 8) are unique, but the results which did emerge are of some interest. The shoulder on the short wavelength side of the Soret band in the 300–380-mμ range required two bands in absorption and two in circular dichroism which corresponded very well in terms of position and width. The peaks are found at 332 and at 357 mμ. In absorption there was a requirement for a band at the long wavelength side of the Soret band which has no counterpart in the CD curve. The remaining range falls within what must be considered the Soret band. Resolution of the Soret band proves interesting. The signs of the bands alternate. Also, there are positive and negative pairs on the basis of band width, though adequate resolution could be achieved without band width pairing. The somewhat surprising feature is that the resolution of the ferriheme undecapeptide curve gives rise to a pattern of bands which markedly resembles the set of curves one expects by inspection of the ferroheme undecapeptide curve (Figure 6), that is, the short wavelength negative band which is obvious in the CD of the reduced heme peptide at about 405 mμ is not apparent in the oxidized heme peptide until the curve has been resolved into Gaussian functions and a negative function is found at 389 mμ. The wavelengths, rotational strengths, oscillator strengths, and anisotropies of each resolved Gaussian are given in Table I for the ferriheme undecapeptide. The resolved curves in Figures 7 and 8 are the absorption and CD curves for the ferriheme undecapeptide (reported in Figure 5) at 3×10^{-4} *M* and 10°. The $\Delta\lambda$ from the longest wavelength to the shortest wavelength band, which comprise the Soret band is 27 mμ for the ferriheme undecapeptide and approximately 20 mμ for the ferroheme undecapeptide. With these values one can approximate a maximal distance between hemes[19,20] and compare this value with what might be expected from inspection of

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Table I. Critical Values for Resolved Gaussian Curves, Ferriheme Undecapeptide (3×10^{-4} *M*, 10°)

Wavelength of maximum	332	357	389	402	408	416	432
Molar extinction coeff $\times 10^{3}$	8	15	22	38	62	19	9
Dipole strength (Dk) $\times 10^{-36}$	6.8	12.1	14.8	25.0	22	6.6	73
Molar ellipticity $\times 10^{3}$	−15	−12	−11	125	−143	36	0
Rotational strength (Rk) $\times 10^{-40}$	9.5	7.5	5.6	65	39	9.2	0
Anisotropy (Rk/Dk) $\times 10^{-4}$	1.4	0.6	0.4	2.5	1.8	1.4	0

the molecular models. A value of 10 A is obtained for the heme–heme distance in the oxidized heme peptide aggregate and 13 A for the reduced aggregate. These maximal values compare quite favorably with those expected from molecular models.

A complex Soret–Cotton effect is not unique to aggregated heme groups. Under conditions where cytochrome *c* is monomeric a complex Soret–Cotton effect is observed.[21-24] The complexity in cytochrome *c* may be ascribed to a steric removal of the degeneracy of the Soret band. It is important to note that the monomeric heme undecapeptide exhibits a relatively simple curve (Figures 5 and 6) and, more significantly, that the difference absorbance studies showed a hyperchromism and a long wavelength shift. Thus, independently of the complexity of the optical rotation patterns, the absorption studies support the presence of heme–heme interaction potentials in the aggregate. Furthermore, the complex CD curve observed for the aggregate in Figure 6 unequivocally demonstrates the presence of at least three discrete transition energies within the Soret band. Removal of the degeneracy of a doubly degenerate band would lead to two bands of opposite sign. Also, the bands for the ferroheme undecapeptide aggregate are likely four in number and are of alternating sign, which is to be expected in terms of reciprocal relations[23] when transitions are coupling to give rise to rotational strengths. It would seem evident that exciton resonance interactions and dispersion force interactions are operative between the hemes within the aggregate. Clearly the complexity of the Soret transition of the heme peptides is dependent on the aggregated state and any similarities which may superficially exist between the complex Soret–Cotton effects of the heme undecapeptide and cytochrome *c* cannot be construed to mean that steric orientation of the heme with respect to peptide chain is virtually unchanged.[25]

The major intent of this work is to allow discriminating discussion and interpretation of data on systems of more direct biological interest. In particular there are applications to the question of heme–heme interaction in hemoglobin and to the possibility of juxtaposed heme groups in the active systems of the electron-transport chain. Simple Soret–Cotton effects have been observed by Frankel and Doty in their ORD studies of hemoglobin.[26] Absence of complex Soret–Cotton effects for oxy- and deoxyhemoglobin shows an absence of exciton resonance interaction and thereby brings into question the presence of substantial polarizability interactions. It would seem that the mode for "heme–heme interaction" is best sought in protein-mediated mechanisms.

With regard to the proximity of heme moieties in components of the electron-transport chain, an enzymatically active preparation of cytochrome oxidase has recently been studied.[27,28] It was found that the Soret band exhibited a relatively simple, positive CD peak in the oxidized state. Upon reduction, however, the Soret band exhibited a complex CD curve of relatively large rotational strengths. The form of the complexity is markedly different from that observed in the CD of cytochrome *c*,[29] and the magnitudes of the curves are several times greater for ferrocytochrome oxidase than for cytochrome *c*. There is, therefore, the distinct possibility that the two heme moieties of reduced cytochrome oxidase are juxtaposed. It is relevant to note that the form of the complex CD curve of ferrocytochrome oxidase, in significant respects, resembles those observed for the heme octapeptide aggregate[18] which also exhibits hypochromism on aggregation. The implication is an orientation of hemes in a more nearly stacked or card pack fashion.

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