Model Systems for Interacting Heme Moieties. II. The Ferriheme Octapeptide of Cytochrome *c*

Dan W. Urry and Jay W. Pettegrew

Contribution from the Institute for Biomedical Research, Education and Research Foundation, American Medical Association, Chicago, Illinois 60610.
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Abstract: In absorption the Soret band of the heme octapeptide exhibits a concentration effect in which there is a 50% hypochromism accompanying a 1000-fold increase in concentration. It is concluded that there are large dispersion force interactions between the hemes at high concentrations and that the relative orientation of the hemes more nearly approximates that of a stacked or oblique conformation rather than that of a head-to-tail alignment. In circular dichroism, the concentration effect is seen as a change from a relatively simple Soret Cotton effect at 10^{-6} *M* to a complex Soret Cotton effect at 10^{-3} *M* which can be resolved into four Gaussian functions. The data are taken to indicate the presence of excitation resonance interactions between Soret transitions. Both the concentration effect and temperature effect suggest at least two modes of aggregation giving rise to polymers with different relative heme orientations. Splitting energies obtained from the resolution into Gaussian functions indicate that the heme–heme distances are less than 7 A. In addition to ligand binding van der Waals interactions are considered as possible stabilizing and orienting forces in the heme peptide polymers. The data on and considerations applied to the heme peptides are related to hemoglobin and cytochrome *c* oxidase as a means of further understanding the problem of heme–heme interaction in hemoglobin and as a basis which suggests possible heme proximity in ferrocytochrome *c* oxidase. A mechanism for heme exchange for hemoglobin is proposed.

In the first paper of this series,[1] aggregation of the heme undecapeptide of cytochrome *c* was studied using the methods of absorption spectroscopy and optical rotation. Heme–heme interaction within the aggregate was assessed by examining changes in the properties of the intense Soret band. On aggregation it was seen that the Soret band became hyperchromic and that the Soret Cotton effect became complex. As the ferroheme undecapeptide exhibited four circular dichroism extrema of alternating sign within the confines of the Soret band, it was demonstrated that the complexity of the Cotton effect was not simply a removal of the Soret transition's double degeneracy. Thus in the aggregated state both dispersion force interactions[2,3] and exciton resonance interactions[4] were observed between the heme moieties. It was therefore possible to approximate the relative orientation of heme groups.

If the heme undecapeptide is further digested by trypsin[5,6] a heme octapeptide is obtained which exhibits hypochromism on aggregation.[7] Accordingly the heme octapeptide provides another model system with which to study heme–heme interaction. In this report absorption and circular dichroism data are presented on the heme octapeptide aggregate. This system shows a marked concentration dependence in absorption and circular dichroism (CD), in accord with the optical rotatory dispersion data of Myer and Harbury.[7,8] A 50% decrease in the per heme absorption on going from 1.7×10^{-6} *M* to 1.7×10^{-3} *M* clearly demonstrates the coupling of transitions in one heme with those of another and indicates a stacked or oblique orientation of the heme planes. Simultaneous fitting of the absorption and circular dichroism curves with Gaussian functions yields approximate splitting energies with which maximal distances between heme centers may be determined. As the transition dipole moment of the Soret band is large and the distances between hemes is small the contribution of the quadripole term to the interaction potential is considered. The close proximity of these highly polarizable groups raises the question of van der Waals energy of interaction between hemes. Such energies should be considered as possible stabilizing forces in the aggregate. These considerations may be extended to systems of more direct biological interest. Substantial van der Waals forces have been suggested to exist between hemes in hemoglobin. Recent work on the heme chromophore to 190 mμ allows maximum values to be placed on these forces. For example, calculation of a maximum total polarizability of the heme group limits the magnitude of van der Waals interaction between heme groups. An upper limit of 500 A^3 for the polarizability of the heme moiety may be compared to the polarizabilities required in order that van der Waals forces explain the heme–heme interaction in hemoglobin. The latter is a phenomenological term applied to the facilitated oxygenation of hemoglobin which is expressed by the sigmoid oxygen binding curve. A sigmoid binding curve indicates an interaction between binding sites (hemes in the case of hemoglobin) but does not presuppose a mechanism. The interactions between hemes discussed in this paper are in terms of mechanism, *i.e.*, dispersion force, excitation resonance, and polarizability interactions.

Another application to biology of these studies on model systems is seen in the complexes obtained from the biological electron transport chain of mitochondria.[9] In this enzyme system electrons are passed from the heme of one cytochrome to that of the subsequent cytochrome in the sequence of carriers. One such com-

(1) D. W. Urry, *J. Am. Chem. Soc.*, **89**, 4190 (1967).
(2) I. Tinoco, Jr., *ibid.*, **82**, 4785 (1960); *J. Chem. Phys.*, **34**, 1067 (1961).
(3) W. Rhodes, *J. Am. Chem. Soc.*, **83**, 3609 (1961).
(4) M. Kasha, *Radiation Res.*, **20**, 55 (1963).
(5) C. L. Tsou, *Biochem. J.*, **49**, 362 (1951).
(6) H. A. Harbury and P. A. Loach, *J. Biol. Chem.*, **235**, 3640 (1960).
(7) H. A. Harbury and P. A. Loach, *ibid.*, **235**, 3646 (1960).
(8) Y. P. Myer and P. A. Loach, *ibid.*, **241**, 4299 (1966).
(9) D. E. Green and A. Tzagoloff, *Arch. Biochem. Biophys.*, **116**, 293 (1966).

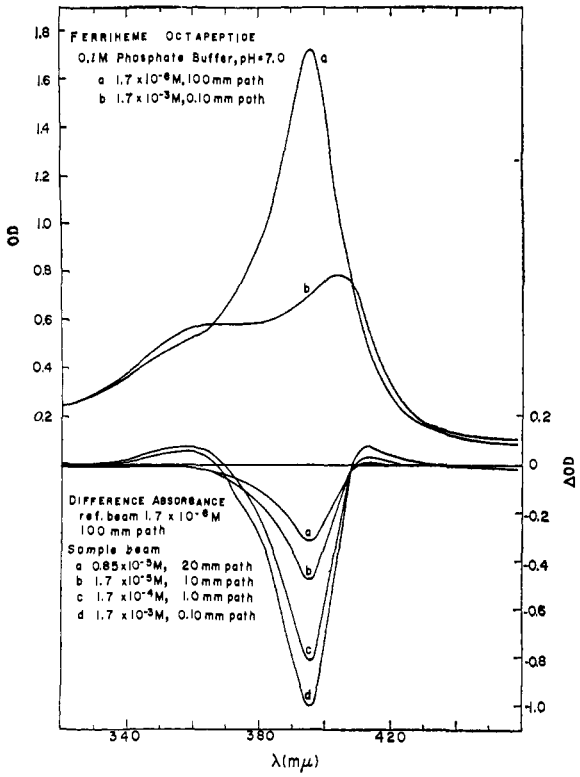

Figure 1. Absorbance and difference absorbance curves of heme octapeptide solutions at different concentrations. There is observed a marked hypochromism associated with increased concentration and an apparent splitting of the Soret band.

plex is cytochrome *c* oxidase which contains cytochromes *a* and a_3. In the light of the model system data, cytochrome *c* oxidase may be studied in an attempt to determine states in which the two heme moieties may be juxtaposed. Comparison of the cytochrome *c* oxidase data[10,11] with that of the heme octapeptide raises the distinct possibility that the heme moieties may be juxtaposed in the reduced state. If excitation resonance interactions are responsible for the complex Soret Cotton effect in ferrocytochrome *c* oxidase then the maximal distance between hemes is 9 A.

Experimental Section

The heme octapeptide was prepared from cytochrome *c* according to the procedure outlined by Harbury and Loach.[6] The buffer was 0.1 *M* phosphate at pH 7. The water used was distilled, deionized, and finally glass distilled. N-Acetyl-L-methioninamide was obtained from Cyclo Chemical Corp., imidazole from Sigma Chemical Co., and dioxane from Burdick and Jackson Laboratories.

Absorption and circular dichroism curves were determined on a Cary Model 14 spectrophotometer and on a prototype circular dichroism attachment built by Cary Instruments for the Cary Model 60 spectropolarimeter, respectively. The CD unit was calibrated using the Cary Model 1401 circular dichroism attachment for the Model 14. The standard used was an aqueous solution of *dl*-camphorsulfonic acid (J. T. Baker, Lot No. 9-361) with an $\epsilon_L - \epsilon_R$ of 2.2 at 190 mμ. Determinations of pH were made on a Radiometer pH meter, Model 25SE. Sample temperatures were maintained with a Haake KT-62 Kryothermat and were monitored with a YSI Model 42SC telethermometer while spectra were being run. The monitoring was achieved by means of a thermocouple inserted through a rubber cap into the cell solution. Curves were resolved into Gaussian functions using the Du Pont 310 curve resolver. Cell path lengths were calibrated using solutions of chromate in 0.05 *N* KOH.

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

(11) D. W. Urry and B. F. van Gelder, to be presented at the "Symposium on Cytochromes," Osaka, Aug 16–18, 1967.

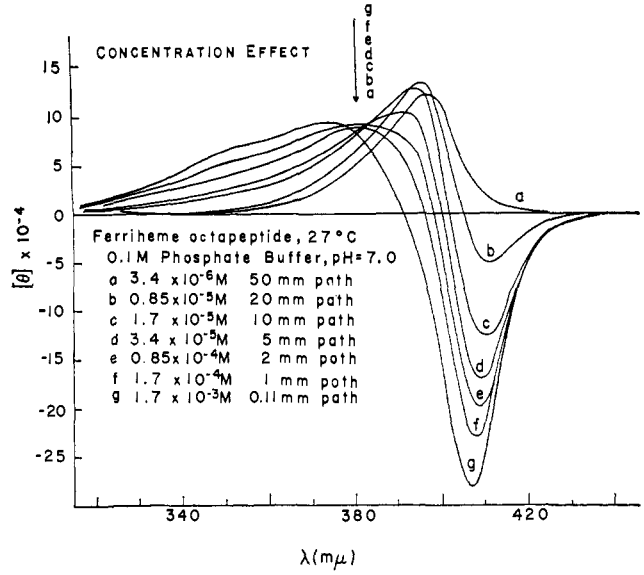

Figure 2. Concentration effect followed by means of circular dichroism. The curves exhibit an increased complexity of the Soret Cotton effect with increases in concentration.

Results

Concentration Effect. The heme peptide solubility, the high absorbance of the Soret band, and the availability of cell path lengths from 100 to 0.1 mm allow ready examination of the heme octapeptide over a 1000-fold variation in concentration. The magnitude of the deviation from Beer's law is seen in Figure 1 where the product of concentration and path length is constant. Difference absorbance curves exhibit a large negative peak at 397 mμ residing between positive peaks at about 360 and 415 mμ. The positive 360-mμ peak is observed when the concentration is 1.7×10^{-4} *M*, but is not observed at 0.85×10^{-5} *M* and 1.7×10^{-5} *M*. The concentration effect as followed by difference absorbance does not indicate a simple equilibrium between two states.

Concentration-dependent variations in the circular dichroism of the heme octapeptide (Figure 2) also demonstrate a multiplicity of states for there are no isoelliptic points; however, at concentrations 1.7×10^{-5} *M* to 1.7×10^{-3} *M* there is the suggestion of an isoelliptic point at about 380 mμ. At the lowest concentration studied and at a temperature of 27° the CD curve is relatively simple with a positive band at 397 mμ in correspondence with the absorption curve (Figure 1). On increasing the concentration a negative peak is introduced at longer wavelengths which becomes increasingly intense and which is found at 408 mμ at the highest concentration. The positive extremum at 397 mμ decreases in magnitude and shifts to shorter wavelengths. Attempts to analyze the concentration effect data in terms of an equilibrium between monomer and polymer indicates that the degree, *n*, of the polymer increases with increasing concentration, the initial value at low concentration being $n = 2$.

Temperature Effects. The concentration effect demonstrates the presence of interactions between heme octapeptide units which in circular dichroism is seen

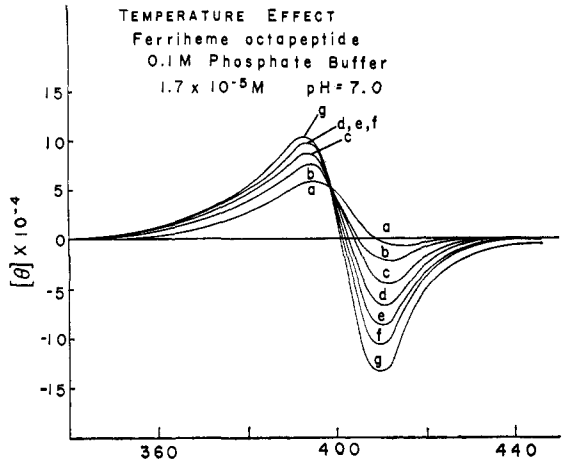

Figure 3. Temperature effect followed by means of circular dichroism. The curves demonstrate a return to a simple Soret Cotton effect with increasing temperature. The curves contain an isoelliptic point indicative of an equilibrium between two species.

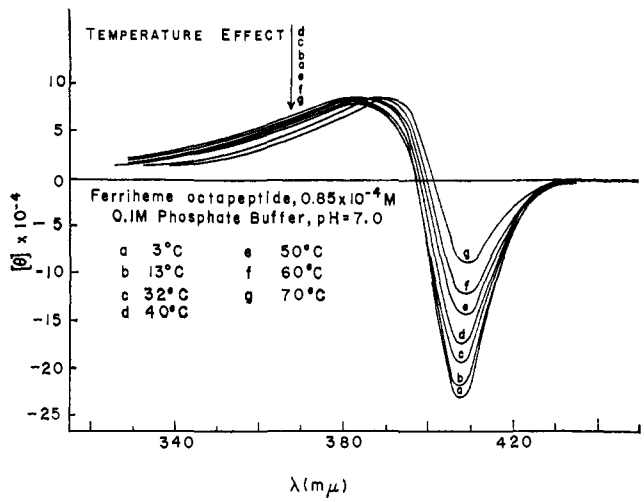

Figure 5. Temperature effect at an intermediate concentration (see text for discussion).

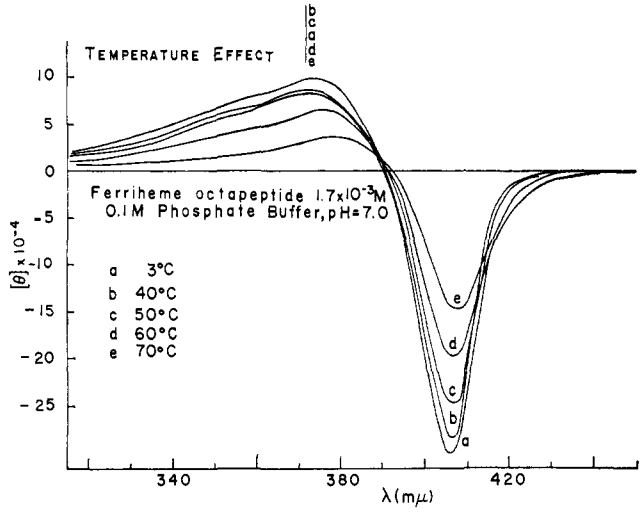

Figure 4. Temperature effect at high concentration (see text for discussion).

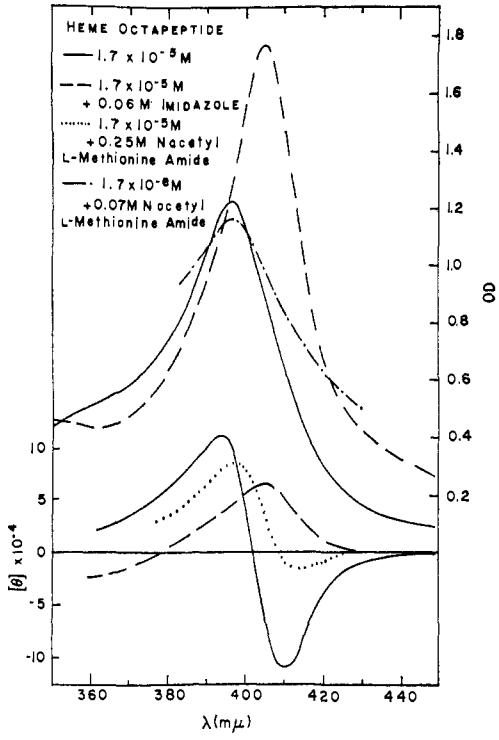

Figure 6. Absorption and circular dichroism demonstrating the effect of added ligands on the magnitude of the absorption and on the complexity of the circular dichroism curves.

as an increase in the complexity of the Soret–Cotton effect. Temperature may also be used to show that the complexity is due to an aggregation. The effect of increases in temperature is to disperse the units comprising the aggregate. As the temperature is increased the circular dichroism curve of a 1.7×10^{-5} M solution (Figure 3) approaches a simple Gaussian function. Both dilution and high temperature favor the monomeric state which is characterized by an approximately Gaussian CD curve. Of additional interest in Figure 3 is the presence of an isoelliptic point suggesting an equilibrium between two states, an aggregate I in equilibrium with monomer. The temperature effect at higher concentration (Figure 4) indicates either that a maximum degree of aggregation has been attained or that the aggregate is of such size that further growth is not discernible in circular dichroism. As the temperature is raised from 3 to 30° there is no detectable change in the CD curve; this is to be contrasted to the large changes seen in Figure 3. Also in Figure 4 is a gross isoelliptic point near 390 mμ which is suggestive of an equilibrium between an aggregate II and monomer. The temperature effect at an intermediate concentration, 0.85×10^{-4} M, is given in Figure 5. This curve suggests an equilibrium between the higher aggregate (II) and aggregate I for a gross isoelliptic point is found near 380 mμ as in the concentration effect (Figure 2) and the ellipticity values at shorter wavelengths do not follow the same order as observed at the negative extremum. The concentration and temperature effects demonstrate a correspondence between complex CD curves and the aggregated state and further show the presence of multiple aggregates which have been grossly divided into aggregate I and aggregate II and which it is to be expected have different modes of polymerization.

Ligand and Solvent Effects. Addition of imidazole to a 1.7×10^{-5} M heme octapeptide solution results in

a relatively simple Soret Cotton effect, in an increase in absorption, and in a shift to longer wavelengths, 397 → 405 mμ, of the Soret absorption maximum (see Figure 6). As it has been shown that the thiol ether group of methionine can form a mixed hemochrome,[12] N-acetylmethioninamide was added. It did effect a loss of complexity of the Soret Cotton effect, but did not cause a shift in the Soret absorption maximum. This suggests that the mode of polymerization in aggregate I might be the thiol ether bridges but raises the question as to the difference in wavelength of absorption maximum in ferricytochrome *c*. Addition of ligands which bind the heme iron brings about a loss of complexity of the Soret Cotton effect, reaffirming that the origin of the complexity is in the interactions within the aggregate. Dioxane also disperses the aggregate (Figure 7) but requires three times the concentration to achieve the same degree of disaggregation as when a thiol ether is employed. The depolymerization effected by dioxane may reflect a ligand effect in which the ether oxygen weakly binds the heme iron or it may reflect a solvent effect indicating the presence of hydrophobic forces in the aggregate. Butyl alcohol–acetic acid (9:1) disperses the aggregate and causes a small red shift. Acetate (pH 7) depolymerizes and causes a small blue shift, 397 → 395 mμ (curve not included). Thus both imidazole and a low dielectric cause red shifts in the Soret maximum.

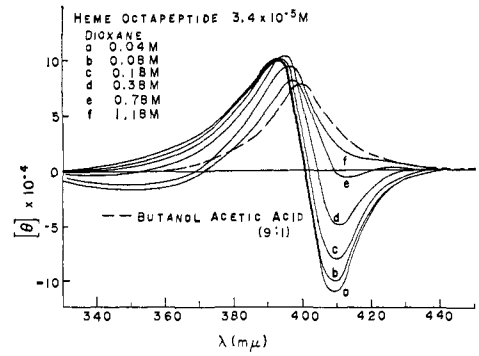

Figure 7. The effect of dioxane and butanol–acetic acid on dispersing the aggregate.

Discussion

Tinoco[2] and Rhodes[3] have shown that hypo- and hyperchromism which occurs when going from a disordered to an ordered state is due to dispersion force interactions in accord with the following equation

$$\left[\frac{F_j}{Nf_j}1 - 1\right] = -K\sum_{i(\neq j)} \frac{f_i}{\nu_i^2 - \nu_j^2} G_{ij} e_i \cdot e_j \qquad (1)$$

where F_j is the oscillator strength of the jth transition of the aggregate or polymer, and division by N, the number of residues in the aggregate, makes the factor, F_j/N, the oscillator strength per residue, a quantity which is calculable without previous knowledge of the number of residues or subunits which comprise the aggregate or polymer. f_j is the oscillator strength of the jth transition for the monomeric state or per subunit of a disordered state. The summation is taken over all the i ($\neq j$) transitions of other residues which are interacting with the jth transition of a given residue. The ν's are the frequencies of the transitions, G_{ij} is a geometric factor from the interaction potential and is usually taken as the dipole–dipole approximation, and e_i and e_j are the unit vectors in the direction of the ith and jth transitions, respectively. As it is usually the long-wavelength band that is being examined ν_i is greater than ν_j. In general, an orientation of transition dipole moments giving rise to a positive G_{ij} results in hypochromism whereas a negative G_{ij} results in hyperchromism when going from the random to the ordered state. A stacked conformation gives rise to a positive G_{ij} whereas a head-to-tail alignment of transition dipoles give rise to a negative G_{ij}. Application of the Tinoco–Rhodes equation to the determination of the relative orientation of heme planes depends on the fact that the polarizability in the direction of the heme plane is much greater than the polarizability perpendicular to that plane. Thus eq 1 can be separated into two summations, one of which is over the transitions with dipole moments in the heme plane and the second over transitions with moments perpendicular to the plane. The former summation would dominate. Thus one is concerned with the interaction of the Soret transition which is in plane with the other in-plane transitions of an adjacent heme moiety.

As the distance between hemes in the heme octapeptide aggregate is less than 10 A due to the structure of the monomer (and may be as close as 4 or 5 A) and as the transition dipole moment, μ, for the Soret band is approximately 8×10^{-18} esu cm, giving an apparent transition dipole length of 1.7 A, it is necessary to consider the effect of adding the quadripole term to the usual analysis of hypo- and hyperchromism data. The geometrical factor, G_{ij}, arising from an interaction potential which includes the quadripole term may be written

$$G_{ij} = D_{ij} + Q_{ij} \qquad (2)$$

$$D_{ij} = \frac{1}{|R_{ij}|^3}\left[e_i \cdot e_j - \frac{3(R_{ij}\cdot e_i)(R_{ij}\cdot e_j)}{|R_{ij}|^2}\right] \qquad (3)$$

$$Q_{ij} = \frac{3}{2|R_{ij}|^5} \times \left[R_{ij}\cdot e_j(e_i \cdot r_i - 2e_i \cdot r_j) - (R_{ij}\cdot e_i)(e_j \cdot r_j - 2e_j \cdot r_i) + \frac{5(R_{ij}\cdot r_j - R_{ij}\cdot r_i)(R_{ij}\cdot e_i)(R_{ij}\cdot e_j)}{|R_{ij}|^2}\right] \qquad (4)$$

where R_{ij} is the distance from the origin of the ith transition to that of the jth transition and D_{ij} and Q_{ij} represent the dipole and quadripole contributions, respectively. The quadripole term contains the factor $R_{ij}\cdot e_i$ and $R_{ij}\cdot e_j$. For the stacked conformation R_{ij} is perpendicular to e_i and to e_j such that the dot products containing R_{ij} are zero and the quadripole term is zero. For the head-to-tail conformation the quadripole term becomes $(1/|R_{ij}|^3)[3(|r_j| - |r_i|)/R_{ij}]$ in which case the quadripole term is positive whenever $|r_j| > |r_i|$ and the dipole term is $(-2/|R_{ij}|^3)$. However $|r_j| - |r_i|$ would have to be greater than 2, which is unlikely, and the distance less than 3 A, which is impossible, before a hypochromism for the jth transition might be expected from interaction with a second transition in a head-to-tail alignment. Thus consideration of the quadripole

(12) H. A. Harbury, J. R. Cronin, M. W. Fanger, T. P. Hettinger, A. J. Murphy, Y. P. Myer, and S. N. Vinogradov, *Proc. Natl. Acad. Sci. U. S.*, 54, 1658 (1965).

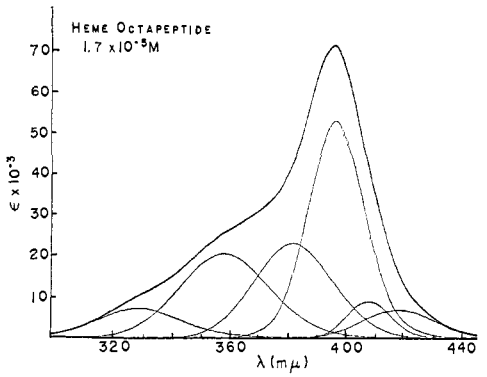

Figure 8. Resolution of the absorption data into component Gaussian functions which simultaneously fit the circular dichroism data as well as absorption and circular dichroism data at higher concentration.

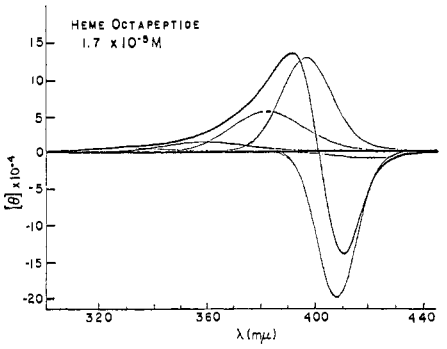

Figure 9. Resolution of the circular dichroism data into component Gaussian functions.

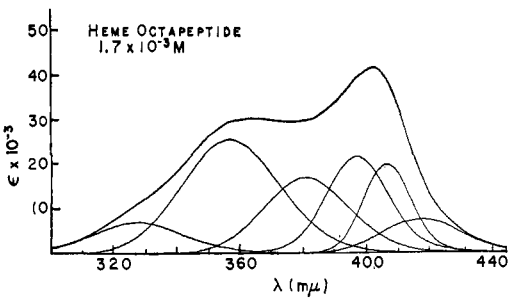

Figure 10. Resolution of absorption data at higher concentration into component Gaussian functions which simultaneously fit the circular dichroism data.

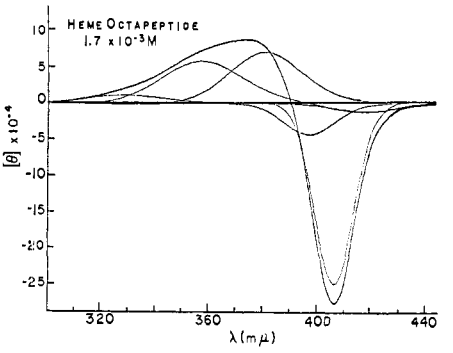

Figure 11. Resolution of circular dichroism data into component Gaussian curves which simultaneously fit the data in Figures 8, 9, and 10.

term in addition to the dipole term does not alter the analysis of the stacked conformation and allows the statement that the large hypochromism attending aggregation of heme octapeptide subunits indicates that the heme planes are oriented in a more nearly stacked than head-to-tail conformation.

The presence of such large dispersion force interactions foretell the presence of large exciton resonance interactions involving the Soret transition. The Soret band is the largest band in the accessible range of heme electronic transitions, a wavelength range which includes transitions in the near-infrared, visible, and ultraviolet to 175 mμ. Since interactions of the Soret transition dipole moment with transitions of lesser oscillator or dipole strengths and of substantially removed frequencies give rise to large hypochromism, as is observed in the dispersion force interactions, then surely there must exist large interaction potentials between Soret transitions which would result in observable exciton splitting in the heme octapeptide aggregate. Observation of a complex Cotton effect occurring in the aggregated state may be taken as an expression of the exciton splitting. A molecular aggregate, when dissymetric, results in split transitions with rotational strengths of opposite sign.

An assessment of the magnitude of the interaction potential may be obtained by resolving the absorption and circular dichroism curves into component Gaussian functions. Our approach is as outlined in the first paper of this series[1] and the instrument used is the Du Pont 310 curve resolver. In the present example it has been possible to simultaneously fit the CD curves of 1.7×10^{-5} M and 1.7×10^{-3} M solutions, as well as the corresponding absorption curves, with a common set of Gaussian functions. The resulting curves are given in Figures 8–11. The critical values for the resolved Gaussian curves are given in Table I for the concentration of 1.7×10^{-5} M and in Table II for the 1.7×10^{-3} M solution. Dipole strengths were calculated using the expression[13]

$$D_i \simeq 1.63 \times 10^{-38}(\epsilon_i^0 \Delta_i/\lambda_i) \quad (5)$$

where ϵ_i^0 is the molar extinction and λ_i is the wavelength corresponding to the band maximum. Δ_i is the half band width at ϵ_i^0/e. Rotational strengths were calculated using the approximation[13]

$$R_i \simeq 1.23 \times 10^{-42}(\theta_i^0 \Delta_i/\lambda_i) \quad (6)$$

where θ_i^0 is the molar ellipticity at the band maximum and Δ_i and λ_i are as defined in eq 5. Anisotropy is the ratio of rotational strength to dipole strength.

Analysis of the concentration effect (Figure 2) indicates that approximately half of the heme octapeptide units are in the aggregated state at a concentration of 1.7×10^{-5} M. As 397 mμ is the extremum of the monomer (Figures 1, 2, 3, and 6) the new bands at 408 and 382 mμ (Figures 8 and 9 and Table I) represent the splitting of the Soret band and give a measure of the interaction potential in aggregate I. This would be a splitting energy of 1.7×10^{-13} erg. The other bands at 418, 358, and 328 mμ are of considerably lower rotational strengths and anisotropy. As was pointed out in the Results section, the 1.7×10^{-3} M solution repre-

(13) A. Moscowitz, "Optical Rotatory Dispersion," C. Djerassi, Ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1960, p 150.

Table I. Critical Values for Resolved Gaussian Curves[a]

Wavelength of extremum, mμ	328	258	382	397	408	418
Molar extinction coefficient × 10^3	7.1	20.2	22.8	52.8	8.7	6.7
Dipole strength (D_i) × 10^{-36}	7.3	20.1	18.6	29.7	3.58	4.44
Molar ellipticity θ_i^0 × 10^3	6.6	14.2	55.2	130	−199	−9.2
Rotational strength (R_i) × 10^{-40}	5.1	10.5	34.0	55.1	−61.8	−4.6
Anisotropy $\lvert R_i/D_i \rvert$ × 10^{-4}	0.7	0.5	1.8	1.8	1.7	1.0

[a] Ferriheme octapeptide, 1.7 × 10^{-5} *M*.

Table II. Critical Values for Resolved Gaussian Curves[a]

Wavelength of extremum, mμ	328	358	382	397	408	418
Molar extinction coefficient × 10^3	7.0	25.3	17.2	21.6	19.6	7.3
Dipole strength (D_i) × 10^{-36}	7.2	25.1	14.0	12.2	8.07	4.83
Molar ellipticity θ_i^0 × 10^3	11.8	56.8	69.7	−44.7	−250	−12.6
Rotational strength (R_i) × 10^{-40}	9.1	42.6	42.8	−18.9	−77.6	−6.3
Anisotropy $\lvert R_i/D_i \rvert$ × 10^{-4}	1.3	1.7	3.1	1.6	9.6	1.3

[a] Ferriheme octapeptide, 1.7 × 10^{-3} *M*.

sents a concentration limit at which either the aggregate has reached a maximal size or further growth does not alter optical properties being studied. This concentration has been taken to represent a second aggregate. Aggregate II is characterized by an increase in absorption at 358 mμ (Figures 1 and 10) and a correspondingly greater increase in the rotational strength of the 358-mμ band (Figure 11 and Table II). As may be seen in Table II there is a fourfold increase in the positive rotational strength of the 358-mμ band while there is increased negative rotational strength in the 408- and 397-mμ bands. ΔR for the 397-mμ band of −74 represents a dramatic change from the higher concentration. Taking the negative 397- and 358-mμ bands as arising in part due to aggregate II a splitting energy of 2.7 × 10^{-13} erg is obtained.

If a dipole interaction potential is assumed, a maximal distance may be calculated between heme moieties within the aggregate, that is

$$R_{ij} < \left[\frac{|\mu_i||\mu_j|2}{V}\right]^{1/3} \tag{7}$$

With the splitting energies (interaction potentials given in the preceding paragraph) the distance between hemes is calculated to be less than 6 A in aggregate I and less than 5 A in aggregate II. These are not true maxima for the quadripole term has not been considered. It is possible however to assess the extent of the dipole approximation in this case. Should only half of the interaction potential be due to the dipole term then the maximal distance would be $2^{1/3}$ or 1.26 times the values given above. As the quadripole term is not expected to exceed the dipole term in magnitude it is reasonable to take maximal heme distances for the heme–heme configuration in aggregate I to be less than 7 A, and as less than 6 A for the heme–heme distance in the configuration called aggregate II.

The very close distances indicated above raise the possibility that the aggregate might be stabilized in part by van der Waals forces. The heme chromophore has recently been characterized to 240 mμ and approximated to 190 mμ.[14] With this information and the sum rule,[15,16] a maximum total polarizability can be calculated for the heme group. Using a reasonable value for the ionization potential of the heme group and considering a coplanar arrangement of hemes (a configuration that favors van der Waals forces) a maximum interaction energy arising from these forces can be obtained. The total polarizability of a molecule in a static field may be written

$$\alpha = \sum_i \alpha_i = \frac{e^2}{4\pi^2 mc^2} \sum_i f_i \lambda_i^2 \tag{8}$$

where e, m, and c are the electronic charge, the mass of an electron, and the velocity of light, respectively. The oscillator strength, f_i, is calculated for an absorption band by the expression

$$f_i = 4.33 \times 10^{-9} \epsilon_i \Delta\nu \tag{9}$$

where $\Delta\nu$ is the band width in cm^{-1} measured at half the band maximum and ϵ_i is the molar extinction coefficient. The sum rule[15,16]

$$\sum_i f_i = n \tag{10}$$

states that a summation over all the oscillator strengths of a group is equal to the number of electrons, n, in the group. Our approach to calculating a maximum total polarizability of a heme moiety is to take the hemochromogen spectrum of the heme undecapeptide of cytochrome *c* as representative and complete the summation in eq 8 from 600 to 200 mμ. By so doing, some of the allowed oscillator strength will have been utilized. If the remainder is placed at 190 mμ a maximum value is obtained, *i.e.*

$$\alpha < \frac{e^2}{4\pi^2 mc^2}\Big[f_{550}\lambda^2_{550} + f_{510}\lambda^2_{510} + f_{418}\lambda^2_{418} + f_{325}\lambda^2_{325} + f_{277}\lambda^2_{277} + (n - \sum_{i=550}^{200} f_i)\lambda^2_{190}\Big] \tag{11}$$

Thus it would appear that the total polarizability for the heme group is less than 500 A^3. The heme undecapeptide spectrum from 600 to 1.8 *m* in D_2O exhibits no bands that would significantly alter the above value. As polarizabilities are of the order of the volume of a molecule[17] a value of 500 A^3 is expected to be maximal.

A maximum interaction energy arising from coplanar heme groups can be calculated by the equation

$$E = -\frac{9I}{8R^6}\alpha^2 \tag{12}$$

where I is the ionization potential which may be taken as 160 kcal/mole[18] and where it has been assumed that all transitions are in the heme plane. The closest distance of approach of heme centers with a coplanar restriction is about 8 A. Using these values E is less than −170 kcal/mole. The enthalpies for heme octa-

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(15) W. Kuhn, *Z. Physik*, 33, 408 (1925).
(16) W. Thomas, *Naturwissenschaften* 13, 627 (1925).
(17) H. Eyring, private communication.
(18) R. M. Hedges and F. A. Matsen, *J. Chem. Phys.*, 28, 950 (1958).

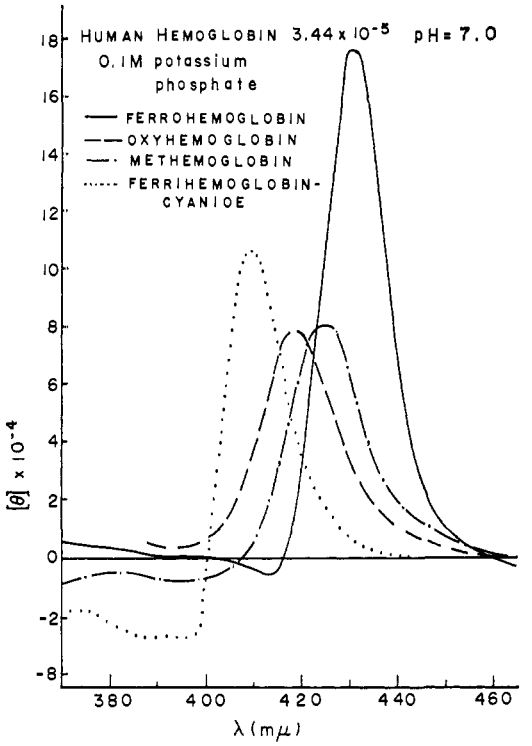

Figure 12. Circular dichroism data on the Soret band of hemoglobin. See text for discussion. Ellipticities are plotted on a per heme basis.

peptide aggregates are −15 to −30 kcal/mole. If the true value of E is lower by more than an order of magnitude it could still be a significant interaction in heme aggregates. The heme octapeptide aggregates, however, are in a stacked conformation rather than the coplanar or head-to-tail arrangement assumed here. It is the heme undecapeptide aggregate for which the above calculation would be most relevant. The above interaction energy may be significant enough to aid in the head-to-tail alignment indicated by the hyperchromism attending aggregation of the heme undecapeptide.

It would appear that the maximal value calculated above is at least an order of magnitude too large. It is now of interest to note what the value of E might be if the hemes were 25 A apart as is the case for the closest pair in hemoglobin.[19] At a distance of 25 A $E < -0.2$ kcal/mole. If it is correct that the magnitude of the maximal value is at least an order of magnitude too large, then a reasonable value to expect would be 0.02 kcal/mole. Thus it would seem unlikely that van der Waals forces could be responsible for the more than 1 kcal/mole interaction which Pauling indicated as necessary for heme–heme interaction,[20] or the better than 20,000 A^3 polarizability which Libby indicates is required,[21] or the approximately 3 kcal/mole free energy of interaction calculated by Wyman.[22]

The circular dichroism curve of oxyhemoglobin in the Soret region is nearly Gaussian. Deoxyhemoglobin has a Soret Cotton effect with a slight negative band on the short-wavelength side which is much more pronounced in ferrihemoglobin (see Figure 12). This complex feature may be due to the removal of the degeneracy of the Soret band, though it bears no resemblance to the CD curve of the Soret band in cytochrome c which is complex due to removal of the degeneracy.[23] The complex feature may be the result of interactions between hemes, as demonstrated in this and the previous paper on heme peptide aggregates. Ferrihemoglobin has been shown to exhibit a heme exchange rate which is ten times greater than that of oxy- and deoxyhemoglobin.[24] Ferrihemoglobin cyanide on the other hand has the lowest exchange rate. The negative band is greatly reduced in the cyanide derivative (Figure 12). There is a coarse correspondence which would suggest that the mechanism of heme exchange involves a displacement or a direct exchange in which the exchanging hemes become juxtaposed. A concentration effect study on deoxyhemoglobin in which the concentration was varied from 3×10^{-3} M to 3×10^{-6} M showed that the slight negative CD band was concentration dependent and that the 430-mμ absorption peak exhibited a hyperchromism as the concentration is raised.[23] A possible explanation of these data is the formation of octamers as suggested by Putzeys and Reijnaers[25] on the basis of their light-scattering data, or an explanation could be the association of $\alpha\beta$ dimers in a manner different from the characteristic tetramer. In particular, the association would be one in which the hemes of different $\alpha\beta$ or of different tetramers were oriented in an approximately head-to-tail fashion with parallel heme planes being slightly displaced from coplanarity. Thus the mechanism of heme exchange would involve a mutual displacement and the rate of exchange would be concentration dependent. One might expect that van der Waals forces are, in part, responsible for the association giving rise to heme exchange. The dipole approximation places the hemes at a maximal distance of 11 A.

Both the heme undecapeptide aggregates[1] and the heme octapeptide aggregates demonstrate that large complex CD bands are the result of interacting heme moieties and that relatively simple bands of lesser amplitude are indicative of monomer. As cytochrome c oxidase contains cytochrome a and cytochrome a_3 it is of interest to determine the possible proximity of these hemes. The products of two different preparations[26,27] have been studied and both yielded the same results.[10,11] A relatively simple positive CD band is obtained for the oxidized state; however on reduction the band becomes complex and the magnitude of the ellipticities is increased. These results have been interpreted to suggest possible heme proximity though it was noted that cytochrome c, which is known to be monomeric, has complex Soret bands in both the oxidized and reduced states. The features which enable comparison of the heme octapeptide aggregate data, in particular, to that of cytochrome oxidase are the large rotational strengths and anisotropies. Both quantities are smaller for hemoglobin and myoglobin and considerably smaller for cytochrome c. The CD data obtained upon

(19) H. Mulrhead and M. F. Perutz, *Nature*, **196**, 633 (1963).
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(21) W. Libby, private communication.
(22) J. Wyman, *Advan. Protein Chem.*, **19**, 224 (1964).
(23) D. W. Urry, R. Purvinas, and A. Ruiter, unpublished data.
(24) H. F. Bunn and J. H. Jandl, *Proc. Natl. Acad. Sci. U. S.*, **56**, 974 (1966).
(25) P. Putzeys and H. Reijnaers, *Nature*, **212**, 506 (1966).
(26) W. W. Walnio, *J. Biol. Chem.*, **239**, 1402 (1964).
(27) L. R. Fowler, S. H. Richardson, and Y. Hatefi, *Biochim. Biophys. Acta*, **64**, 170 (1962).

reduction of the cyanide derivative of cytochrome *c* oxidase is easily understood in terms of exciton resonance interactions.[11] Should the hemes be juxtaposed a maximal heme–heme distance of 9 A can be calculated. An important reservation must be retained and that is that no aggregation studies have been carried out on heme a systems. Work directed toward that goal is presently under way.

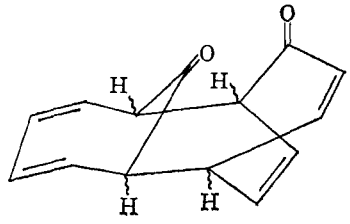
Acknowledgments. The authors wish to thank M. Mednieks for preparing the heme octapeptide, R. Purvinas and A. Ruiter for technical assistance, and Professor G. Guidotti for helpful discussions.

Communications to the Editor

Stereochemistry and Mechanism of a Tropone Photodimerization

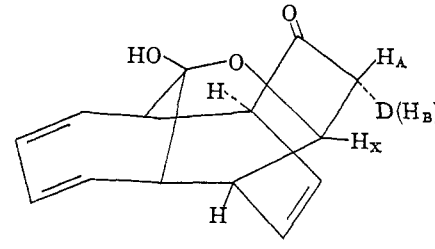
Sir:

We have recently described the formation of a tricyclo[6.3.2.1^{2,7}]tetradeca-3,5,9,12-tetraene-11,14-dione (I) of unspecified stereochemistry as a major product of tropone photodimerization in acetonitrile.[1] This reaction formally represents a (6 + 4) π electron cycloaddition, a process not normally favored for concerted photochemical *cis* addition[2] between two polyene systems.[3] We now report observations which permit assignment of stereochemistry in this series and offer data bearing on the mechanism of the photodimerization process leading to I.



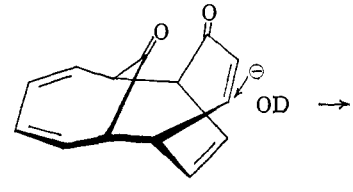
I

Brief treatment of photodimer I with cold dilute NaOD produces two crystalline *hydration products*, mp 201 and 187° dec. The 201° product (ν^{KBr} 3400, 1717 cm^{-1}) retains the diene portion of precursor I but lacks the unsaturated ketone chromophore; it contains one carbon-bound deuterium[4] and one hydroxyl group (by nmr exchange). The nmr of an undeuterated sample shows an ABX pattern with $\delta_A = 2.60$, $\delta_B = 2.87$, $\delta_X = 4.75$; $|J_{AB}| = 17$ Hz, $|J_{AX}| = 5$ Hz, $|J_{BX}| = 1$ Hz.[5] In the deuterated material the B proton signal is absent, and the X proton is a double doublet indicating further coupling of X to a single vicinal proton with $|J| = 7$ Hz. The spectroscopic data agree with the hemiketal structure II which, moreover, rationalizes the observed resistance of the compound to oxidation by chromium trioxide in pyridine.

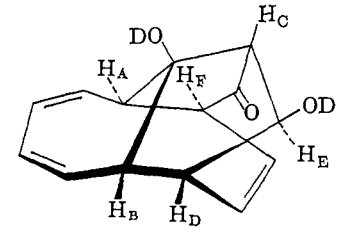


II

The 187° hydration product contains *no carbon-bound deuterium;* it shows ultraviolet absorption similar to II, a single carbonyl maximum at 1748 cm^{-1}, and two hydroxyl groups (nmr). These facts suggest a mechanism wherein attack of deuterioxide on the enone double bond leads to an enolate anion which undergoes internal aldolization to the β-hydroxy ketone III faster than it suffers external deuteron uptake on carbon.



I



III

The 100-MHz nmr spectrum (Figure 1)[6] of the 187°

(1) A. S. Kende, *J. Am. Chem. Soc.*, 88, 5026 (1966).

(2) As originally formulated, the Hoffmann–Woodward rules[3] for concerted cycloadditions apply to *cis* additions; for *trans* cycloadditions, which may be observed in sufficiently flexible systems, a reversal of the usual rules is predicted. We are indebted to Professor R. Hoffmann for calling our attention to this point.

(3) R. Hoffmann and R. B. Woodward, *J. Am. Chem. Soc.*, 87, 2046 (1965).

(4) Mass spectrometric analyses were carried out by the Morgan–Shaffer Corp., Montreal, Canada. All new compounds gave satisfactory combustion analyses and exhibited mass spectrometric fragmentation patterns in accord with the postulated structures. We are grateful to L. Brancone and W. Fulmor for the analytical and spectrophotometric data and to Dr. J. Karliner for interpretation of the mass spectra.

(5) These values were determined at 60 MHz in $CDCl_3$ solution; the δ values refer to parts per million downfield relative to internal tetramethylsilane.

(6) We are grateful to Dr. L. A. Wilson (Rutgers University) for the data of Figure 1, which were measured for a pyridine-d_5 solution (after exchange of active hydrogen) using a Varian HA-100 spectrometer.