AN ABSTRACT OF THE THESIS OF

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Title STUDIES ON HEMATIN a, ITS INTERACTIONS WITH POLY-AMINO ACIDS AND APO-CYTOCHROME c OXIDASE

Abstract approved [redacted]
(Major professor)

Oxidized and reduced hematin a in aqueous solution exhibited anomalous optical rotatory dispersion (ORD) in the wavelength range 500-220 mμ. Dispersion curves of two distinct types were observed depending on the initial mode of solubilization of hematin a. Type I hematin solution exhibited a complex ORD spectrum for the oxidized form (amplitude in molar rotation per dm, 14,000°; apparent cross-over point, 410 mμ) but a relatively simple Cotton effect for the reduced (amplitude, 59,000°; cross-over point, 397 mμ). Analysis of the experimental dispersion curves showed that the oxidized form is composed of at least 4 Cotton effects and the reduced, 3 Cotton effects. Type II hematin solution gave complex ORD profiles for both the oxidized and reduced forms. However, the magnitude of rotation was several fold greater than that of Type I. ORD behaviors are

affected by concentration, pH, sodium chloride, carbon monoxide (CO), Emasol-1130 and organic solvents; these results suggest that the observed ORD spectra are the composite of several factors. They are, inter alia, aggregation, ligand formation, stereochemistry of the porphyrin ring and side chains and the orderly structure or conformation of the molecule.

Both reduced and CO-reacted alkali denatured cytochrome c oxidase exhibited anomalous ORD in the Soret absorption region. The ORD profile showed a deep trough (437.5 mμ, reduced; 435 mμ, CO-reduced) superimposed on the protein backbone rotation. The amplitude was about 15,000° (reduced) and 20,000° (CO-reduced). Heme a complexes of poly-L-lysine and poly-D-lysine, likewise, exhibited anomalous ORD in the Soret region. For heme a-poly-D-lysine (Ave. $\bar{p}$ = 60), the dispersion curve, having a distinct negative extremum at 427.5 mμ, appeared to be qualitatively similar to that of the denatured enzyme. Carbon monoxide induced a blue shift of the Cotton effect. Complexes of poly-L-lysine (Ave. $\bar{p}$ = 578) with or without Emasol-1130, showed quite different ORD. Without Emasol it exhibited an asymmetric positive Cotton effect (maximum, 435 mμ; minimum, 422.5 mμ) which became symmetrical when the complex was reacted with CO. The amplitude was 113,000° (reduced) and 128,000° (CO-reduced) respectively. The complex in Emasol gave a couplet-type ORD having a dominant peak at 436 mμ flanked by two small troughs. The profile was reduced to a simple positive Cotton effect in carbon

monoxide (maximum 427 mμ, minimum, 412 mμ). The cross-over point (421 mμ) was close to the absorption maximum (424 mμ). Protoheme-poly-L-lysine gave a negative Cotton effect (minimum, 435 mμ; maximum, 418 mμ and cross-over point, 429 mμ). Maximum amplitude was 650,000°.

Spectropolarimetric titrations of heme a-poly-L-lysine without Emasol (system A), gave a maximum amplitude at lysine residues to heme (L/H) ratio of 50.3 but gradually decreased at higher L/H ratio. Similar titration behavior was observed for system B (complex in Emasol). However, the CO-treated system B showed the normal titration behavior. The amplitude remained at maximum value even at high lysine concentration. Similarly, the titration curve of protoheme-poly-L-lysine did not show any abnormality.

These ORD results are explained in terms of heme-polypeptide and heme-heme interactions. Optical rotations in system A suggest strong dispersion interactions among heme molecules localized along the polypeptide chain. The couplet-type ORD observed for heme a-poly-D-lysine and heme a-poly-L-lysine in Emasol-1130, indicates strong heme-heme and heme-polymer interactions. From these observations, the heme groups in alkali denatured cytochrome c oxidase are deduced to be closely aligned but oriented differently as compared to those in synthetic complexes.

The preparation of an apo-cytochrome c oxidase and its interactions with hematin a are also described.

STUDIES ON HEMATIN a, ITS INTERACTIONS WITH POLY-AMINO ACIDS AND APO-CYTOCHROME c-OXIDASE

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Dedicated to my wife and to my parents.

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STUDIES ON HEMATIN a, ITS INTERACTIONS WITH POLY-AMINO ACIDS AND APO-CYTOCHROME c OXIDASE

GENERAL INTRODUCTION

The metabolism of oxygen is, by any measurement, one of the most profound and unique chemical events that has occurred in nature. Its universality is shown by its occurrence in all aerobic living systems---plants, animals and microbes. A memorial record for the early history has been recently published (26). In the mammalian organisms, oxygen is activated and subsequently converted to water through the action of the most important terminal respiratory enzyme system known as the cytochrome c oxidase. This enzyme system distinguishes itself from other oxidase and oxygenase systems by its ability to conserve energy (oxidative phosphorylation) during electron transfer and oxidation. In the past decade, extensive efforts have been concentrated on the purification and characterization of the enzyme (12, 17, 29, 76). It is now generally accepted (29, 69) that the functional unit of the enzyme is composed of an equal amount of cytochrome a and cytochrome a_3. The copper to heme ratio is 1.

Despite the wealth of information now available on the structural aspects of hematin a (12, 17, 19, 20, 39, 42, 48, 79) which is the prosthetic group of cytochrome oxidase, its optical activity which is to be expected from the asymmetric structure of the heme molecule, has

never been demonstrated (33, 42, 49). This thesis describes the measurement of optical activity of hematin a in aqueous medium. The complexes formed between heme a and polylysine are also characterized by optical rotatory dispersion measurements and served as model for the alkali denatured cytochrome c oxidase. The interaction between hematin a and an apo-cytochrome c oxidase preparation has also been studied.

Extensive reviews (10, 39, 42, 45, 56) and symposia (12, 14, 17, 29, 55) organized around the general theme of redox enzyme systems had been published in recent years. Of particular value are the publications of the classical studies on cytochromes by Professor D. Keilin (26) and on heavy metals as prosthetic groups by Professor Otto Warburg (70).

1. OPTICAL ROTATORY DISPERSION OF HEMATIN a

INTRODUCTION

It is generally believed that hematin a is the prosthetic group of cytochrome oxidase, i.e. of cytochrome a and cytochrome a_3. As early as 1924, Warburg (70) predicted the presence of a formyl group in hematin a. The prediction has been experimentally verified by many workers (cf. 39). In Warburg's laboratory, chemical studies of hematin a were accelerated after its crystallization (71). Although other investigators failed to crystallize hematin a (for example 19, 20, 39), a number of preparations (27, 38, 47, 48, 50) were obtained with suitable purity for characterization. However, definite progress was not made until 1963 when Grassl, Augsburg, Coy and Lynen (19) provided evidence for the structure from their studies on degradations of the samples of 95% purity and partial synthesis of some derivatives. The main contributions by Grassl, et al. (19, 20) are on the identification of the two alkyl side chains on the porphyrin, the placement of a vinyl group at position 4 and the elucidation of the long alkyl group ($C_{15}H_{31}$-) at position 2. Caughey and co-workers (8, 9, 79), however, have raised doubt about the structure proposed by Grassl, et al. (19, 20) because they have found a hexosamine group also existing in side chain 2, probably through an ether-linkage to the 2-α carbon atom. This structure is actually not impossible.

With the Pauling-Corey-Kolten model, we[1] have been able to build such a structure for hematin a. The resulting molecule has all the hydroxy groups of the pyranose ring aligned to one side and the nitrogen atom is coordinated to the iron atom. The molecule becomes extremely compact and stressed. In spite of these uncertainties which were not resolved even as late as in the recent Cytochrome Symposium in Kobe (University of Tokyo Press, in press), the primary structure of hematin a in general may be represented as in Figure 1.

We are more interested in the secondary structure of hematin a. It seems inconceivable that a molecule as large as hematin a with a long hydrophobic side chain in addition to other functional groups can exist without an orderly conformation, even disregarding the idea of a hexoseamine group. If the orderly conformation does in fact exist in addition to its asymmetric carbon atoms, hematin a should be optically active and multiple Cotton effects should be expected. However, the lack of optical activity was unanimously reported (33, 42, 49) prior to recent findings (77). Now we know that the optical inactivity reported in earlier studies (33, 42, 49) was evidently due to the solvents

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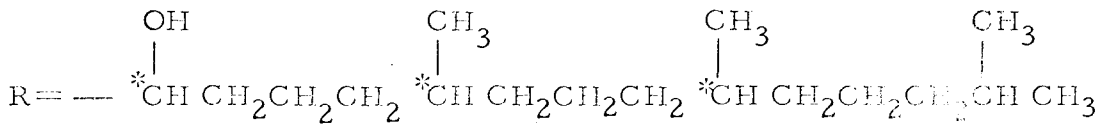
[1]The courtesy of Dr. Philip Wilcox, University of Washington, who put his enormous facilities at our disposal for making this model and other hematin a derivatives with ligands of small molecules and of macromolecules, is gratefully acknowledged.

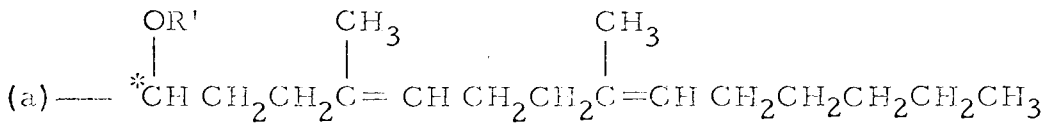
employed in the measurements. Hematin a is relatively insoluble in aqueous media. Consequently, workers usually use pyridine or detergent to facilitate solution. These solvents abolish the optical activity of the aqueous hematin solution. It should be emphasized that the hematin a samples used in our laboratory are prepared from purified cytochrome oxidase of high enzymatic activity which is free from other hematins, in contrast to preparations used by other workers who directly isolated the hemes from muscle mince (e. g. 9, 19, 20, 71, 79).

This section of the thesis reports in detail the optical activity of hematin a under various conditions. The origin of the optical activity and the complications due to aggregation of the molecule are discussed.

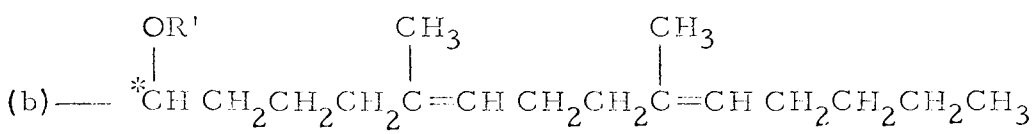
Figure 1. Structure of hematin a. According to Grassl and co-workers (19, 20).



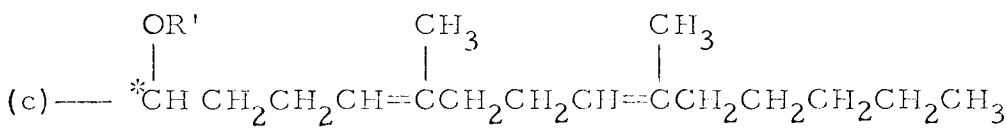
According to Caughey, et al. (9), R may be one of the following:



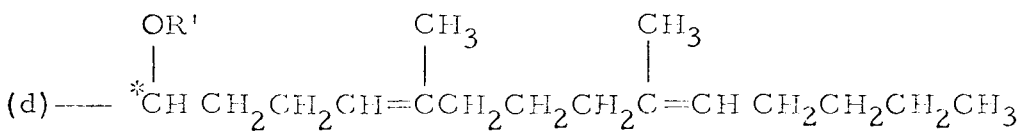
(a)



(b)



(c)



(d)

and R' = $C_6H_{11}NO_4$. (*), asymmetric carbon atom.

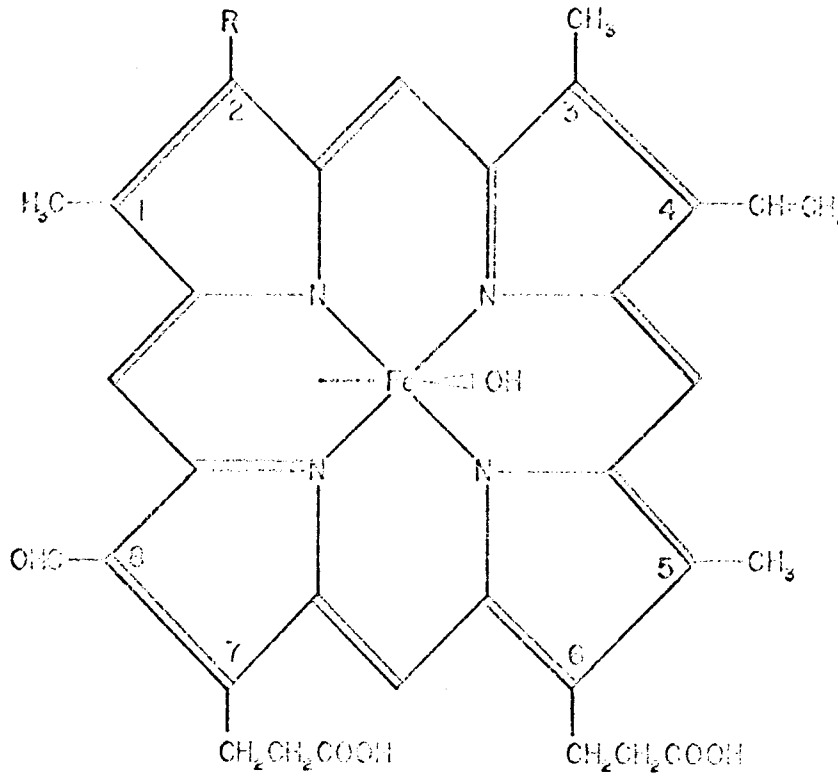


Fig. 1

EXPERIMENTAL PROCEDURES

Materials

Hematin a -- Hematin a was prepared from purified cytochrome oxidase with enzymatic activity between 70 and 100 electron equivalents per mole of enzyme (in terms of hematin a) per second, according to the method described previously (62) except that three additional extractions with methanol-chloroform (1:2, v/v) were introduced prior to acid-acetone extraction. Special precautions in conducting the preparation at low temperatures (0-4°) and in avoiding exposure to strong light were always taken. The dried hematin a was stored at about -25° in a vacuum dessicator protected from light.

Crystalline hemin-chloride (protohematin-HCl) was purchased from Mann's Research Lab. Emasol-1130 (polyoxyethylene-Sorbitan-monolaurate), a nonionic detergent, was kindly supplied by Kao Soap Company, Tokyo. Other chemicals of highest purity were obtained from commercial sources.

Methods

The solution of hematin a was prepared in two ways. Type 1: The solid hematin was dissolved in a minimal amount of 0.1 N NaOH

and the solution was diluted with 0.1 M phosphate buffer (KH_2PO_4-Na_2HPO_4), pH 7.0, to give the pH of the stock solution at 9.0-9.5. It was stored at 4° for not more than six hours. For use, aliquots were diluted with phosphate buffer to give the desired concentrations of hematin a in 0.05 M Sørenson phosphate buffer, pH 7.7-8.0.

Type II: The solid hematin a was dissolved in 0.01 N NaOH. The solution was then immediately diluted to the appropriate concentrations with phosphate buffer at 0.05 M, pH 7.7-8.0.

Hematin a concentration was determined by the pyridine hemochromogen method (16), and that of heme iron by direct chemical analysis (28). Reduction of samples was accomplished by addition of a minimum amount of sodium dithionite.

Thin-layer chromatography -- Sample of hematin a as such and after acid hydrolysis were tested by thin-layer chromatography for possible contamination. Hematin a was hydrolyzed in 5.7 N HCl in a sealed tube at 110° for 20 hours. The HCl was removed in vacuo as the usual practice for the removal of HCl in protein hydrolysates. Appropriate aliquots were applied to silica gel plates of 0.5 mm thickness. They were developed in appropriate solvent systems. Butanol-acetic acid-water (4:1:1, v/v) was used for separation of amino acids and peptides, petroleum ether (b.p. 30-60°)-ethyl ether-acetic acid (70:30:1, v/v) for lipids, and chloroform-methanol-water

(65:25:4, v/v) for phospholipids. The time used for the developments ranged from 30 to 160 minutes. The plates were then air dried at room temperature. Ninhydrin reagent was used for the detection of amino acids and peptides, and Zinsadse reagent (15) for phospholipids, and 50% ethanol in sulfuric acid for lipids.

Instrumentation -- All pH measurements were made with a Beckmann Zeromatic pH meter fitted with a Metrohm electrode (model U) which needs no Na^+ correction at pH <12. Optical rotatory dispersion (ORD) measurements were conducted in a Cary Model 60 spectropolarimeter at 23 $\pm$ 0.5°. The slit-width of the instrument has been programmed for a band width of 15 Å in order to give a constant light intensity over the wavelength range from 185 mμ to 600 mμ. Cells of 50, 20, 10 and 1 mm light path were used. Rotation was calibrated with standard sucrose solution. The observed rotations were converted into molar rotations in the unit of degrees per mole hematin a per liter per decimeter without correction of refractive index. Absorption spectra were measured before and after each ORD run in a Cary Model 14 spectrophotometer and were observed not to have changed.

Sedimentation velocity studies of hematin a solutions were carried out in a Spinco Model E analytical ultracentrifuge at 24,000 and 48,000 rpm at 20°, using a standard cell with a 30 mm aluminum

centerpiece. Schlieren optics, Kodak red sensitive plates type 1-N and a Kodak red filter No. 29 were employed.

RESULTS

Purity of Hematin a Preparations -- The purity of cytochrome oxidase and hematin a has been described in earlier papers (34). Hematin a and its starting material, cytochrome oxidase, contained no other hematins. Results from chromatography of hematin a samples, before and after acid hydrolysis, showed no contaminations of lipids, phospholipids, peptides and amino acids. Direct chemical analysis of heme iron gave values between 5.35 and 5.64% which were equivalent to 90-92% purity for a molecular weight of 935 or 95-97% for 960.

ORD of Hematin a (Type I) -- The ORD profiles of hematin a and its reduced form are shown in Figure 2 and their absorption spectra in Figure 3. It can be noted that anomalous rotations were observed for both the oxidized and reduced forms in regions below 500 mμ. The multiple Cotton effects with broad band width of the oxidized form suggest overlapping of these effects of opposite signs. Upon reduction the solution yielded a more conventional profile resembling a single Cotton effect. The center of the main effect was at 397 mμ with an amplitude of rotation of 59,000°. The magnitude of rotation was found to be dependent upon the experimental conditions, e.g. pH and concentration (vide infra). The inflection

Figure 2. ORD of the oxidized and reduced forms of Type I hematin a in 0.05 M phosphate buffer, pH 7.8. Concentration of hematin a: 191.2 μM, light-path 0.01 dm.

Figure 3. Absorption spectra of the oxidized and reduced forms of Type I hematin a. Other conditions same as in Figure 2.

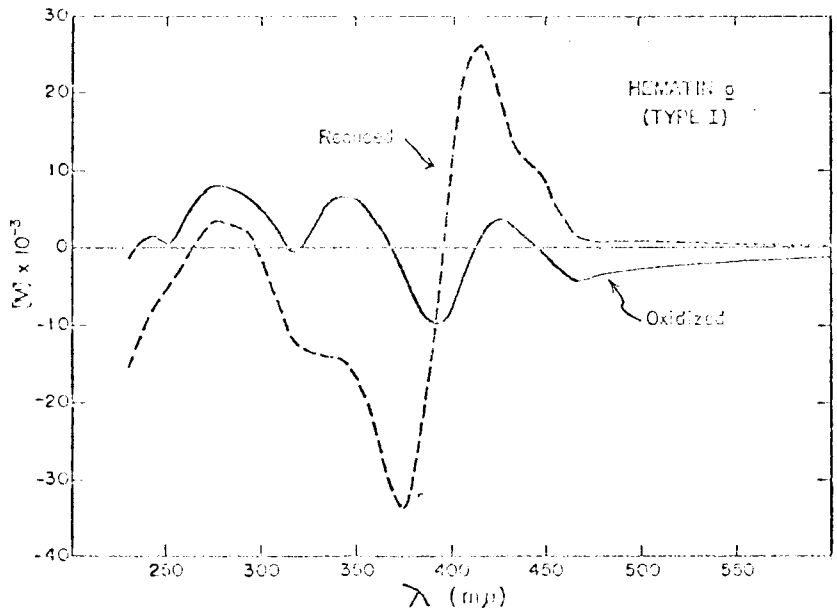

HEMATIN a
(TYPE I)
Reduced
Oxidized
[M] x 10⁻³
λ (mμ)

Fig. 2

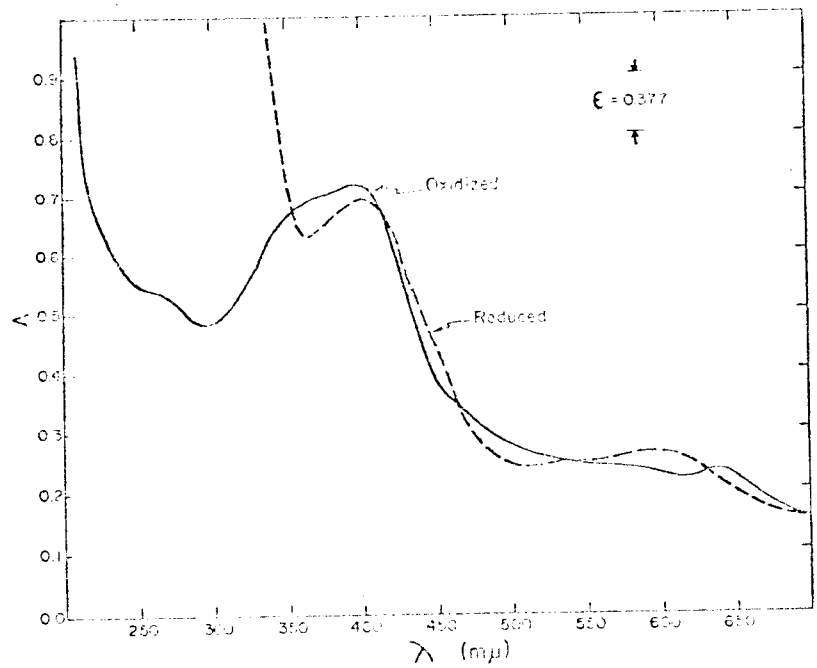

ε = 0.377
Oxidized
Reduced
λ (mμ)

Fig. 3

points of 397 mμ for the reduced and of 410 mμ for the oxidized forms did not exactly coincide with the absorption maxima, viz., 402 mμ for the reduced and 400 mμ for the oxidized forms. This discrepancy might well be attributed to the poor definition of individual transitions within broad bands (cf. Figure 3) and the overlapping of multiple Cotton effects. Table 1 summarizes the ORD parameters obtained by the method of analysis developed by Carver, et al. (7) and adopted by Schellman and King(59). The following phenomenological equations were used for the resolution of the extrinsic Cotton effects:

$$[M]_\lambda = \frac{A'_{193}(193)^2}{\lambda^2 - (193)^2} + \frac{A'_{225}(225)^2}{\lambda^2 - (225)^2} + \sum_i F(\lambda_i, \Delta_i, A_i) \quad (1)$$

and

$$F = \frac{A_i \lambda_i}{\Delta_i} \left[e^{-x^2} \int_o^x e^{t^2} dt - \frac{\Delta_i}{2(\lambda+\lambda_i)} \right] \quad (1a)$$

$$\text{and } X = \frac{\lambda - \lambda_i}{\Delta_i}$$

In this equation, $[M]_\lambda$ is the molar rotation in degree per mole heme a per liter per dm at an operational wavelength λ; A'_{193} and A'_{225} are the Shechter-Blout parameters (7) on the molar basis; Δ_i, A_i, and λ_i are the bandwidth, strength parameter and the center

of the Cotton effect, respectively. For hematin a, the first two terms of equation (1) were taken as zero.

TABLE 1

Parameter of Resolved Cotton Effects of Hematin a

Oxidized	I	II	III	IV
λ (mμ)	408	389	308	325
Band width (mμ)	23	73	22	21
Rotational strength (D. M.)	0.125	-0.36	-0.075	-0.079

Reduced	I	II	III
λ (mμ)	394	308	452
Band width (mμ)	20	35	22
Rotational strength (D. M.)	0.266	-0.18	-0.029

The results of the analysis showed that a minimum of 4 components were resolved to satisfy the observed rotatory dispersion. Indeed, this sort of overlapping Cotton effects could be clearly demonstrated only by this type of computer analysis. The main transitions were at 408 mμ and 389 mμ. These two Cotton effects were of opposite signs and had rotational strength of 0.125 and -0.36 D.M., respectively. The reduced form of hematin a was resolved into 3 components. The main Cotton effect was centered at 394 mμ with a rotational strength of 0.266 D. M.

A pure sample of dipyridyl derivative of hematin a kindly supplied by Dr. Winslow Caughey of Johns Hopkins University Medical School was also tested. After removal of pyridine according to the method developed by Warburg, et al. (72), the solution showed practically the same ORD behavior as described.

ORD of hematin a (Type II) -- ORD profiles of hematin a solutions of Type II are shown in Figure 4. The absorption spectra are similar to those in Figure 3 except for a slight increase in absorption at 610 and 560 mμ for the oxidized form. Again as observed in Type I hematin a solution, the optical activity was dependent upon its oxidation state. In contrast to Type I, however, the oxidized form of Type II showed anomalous rotations with no clear-cut inflection point in the Soret region. The entire curve appeared to have been displaced toward more dextro-rotation. Upon reduction, the ORD spectrum resembled that of Type I except in the 420 mμ region. In this region, there appeared to be another Cotton effect with transition at about 440 mμ. The magnitude of rotation for both the oxidized and reduced forms was several fold higher than that of Type I. A consistent feature between the two types, whether oxidized or reduced, was the similarity of the dispersion curve in the 250-300 mμ region.

The difference in ORD between Type I and II was consistent

Figure 4. ORD of the oxidized and reduced forms of Type II hematin a in 0.05 M phosphate buffer, pH 7.8. Concentration of hematin a: 191.5 μM, light-path: 0.01 dm.

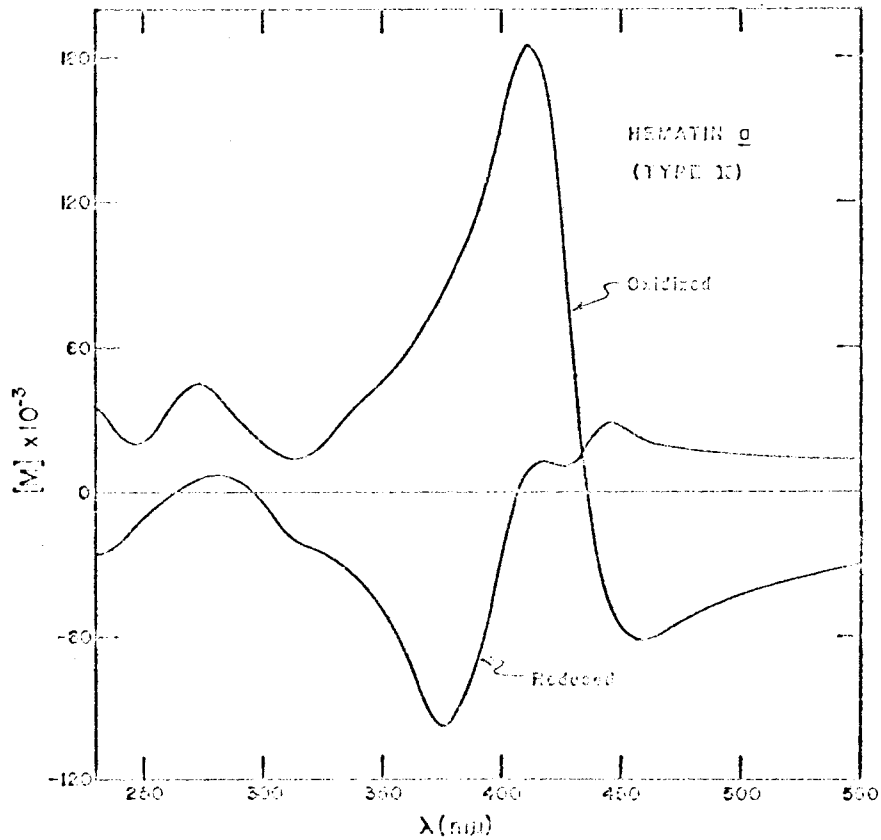

HEMATIN a
(TYPE X)
Oxidized
Reduced
[M] x10⁻³
λ(mμ)
120
60
0
-60
-120
250
300
350
400
450
500
550

Fig. 4

and reproducible for the same batch as well as for different batches of hematin a. Occasionally, dispersion curves intermediate between Type I and II were also observed. The anomalous ORD behavior, the enormous increase in rotation, and the absorption spectra of the solution suggested that Type II hematin a solution probably existed in a highly aggregated form. Indeed, we found that the magnitude of rotation was dependent upon the concentration.

Effect of Concentration -- Measurements were made by varying reciprocally the concentration of hematin a with respect to the light path of the cell so that the absorbance remained approximately of the same values. The effect of concentration on the amplitude of the Cotton effect is summarized in Table II. It may be noted that the amplitudes of rotation of the oxidized form of both types were virtually not affected by concentration; the complete ORD spectra remained practically the same. Likewise, the absorption spectra remained essentially unchanged. In the reduced form, however, the magnitude of rotation for both types was found to be dependent upon the concentration of hematin a. As shown in Table II, the amplitude of rotation increased approximately seven fold when the concentration was raised from about 5 to 250 μM in Type I, and approximately four fold when the concentration was increased from about 3.83 to 190 μM in Type II. These results suggest that aggregation might be involved in the observed optical rotation, particularly in Type II solution.

TABLE II

Effect of Concentrations on the Amplitudes of Apparent Cotton Effects and Absorption Maxima of Hematin a

Hematin a solution			Oxidized			Reduced		
Type	Concentration (μM)	Light-path (dm)	Soret Absorption Maximum (mμ)	ϵ mM^{-1} cm^{-1}	ORD Amplitude $[M]\times10^{-3}$*	Soret Absorption Maximum (mμ)	ϵ mM^{-1} cm^{-1}	ORD Amplitude $[M]\times10^{-3}$*
					(412-385 mμ)*			(415-373 mμ)*
	250	.01	400	37.0	15.2	401	34.5	86.4
I	24.9	.1	399	35.6	15.5	409	28.8	22.5
	5.11	.5	398	33.7	13.3**	410	28.0	12.9
					(412-345 mμ)*			(412-370 mμ)*
	191.5	.01	400	40.3	144	398	37.4	111
II	9.57	.2	398	39.4	130	405	30.5	55
	3.83	.5	398	40.5	133	410	30.1	28

*peak minus trough values in degree per mole of hematin a per liter per decimeter

**425-392.5 mμ

Effect of Sodium Chloride -- Sodium chloride was observed to transform the ORD spectra of Type I hematin a solution in both oxidation states to those similar to Type II hematin a. The conversion was not complete under the conditions studied mainly because high concentrations of NaCl caused precipitation of the hematin. The salt effect was observed to be more prominent in dilute than in concentrated solutions. Figure 5 depicts the effect of NaCl on a dilute hematin a solution. In the absence of NaCl (Curve A), the solution gave the typical Type I dispersion curve. Addition of 0.01 M NaCl (Curve B) displaced the dispersion curve below 440 mμ towards more dextro-rotation. The profile in the wavelength range 500-300 mμ appeared to have been changed; thus, a broad negative extremum became apparent at 460 mμ. The positive extremum at 417.5 mμ increased considerably and was blue shifted to 415 mμ. The most significant feature of the salt effect was the displacement of the 387.5 mμ negative extremum from negative rotation towards positive values. The profile of the dispersion curve below 300 mμ remained unchanged. The amplitude of rotation (417.5 mμ - 387.5 mμ) was increased, in the presence of salt, from 14,800 to 21,000°. The effect of salt on the reduced solution is shown in Figure 6. Curve A represents the dispersion in the absence of salt. Addition of salt (Curve B) decreased the rotation at 417.5 mμ while a new

Figure 5. Effect of sodium chloride on the ORD of Type I hematin a in 0.05 M phosphate, pH 7.8. (A) No NaCl, (B) in presence of 0.01 M NaCl. Hematin a concentration: 25 μM; light-path: 0.1 dm.

Figure 6. Effect of sodium chloride on the ORD of Type I hematin a (reduced form) in 0.05 M phosphate, pH 7.8. (A) No NaCl, (B) in presence of 0.01 M NaCl. Other conditions same as in Figure 5.

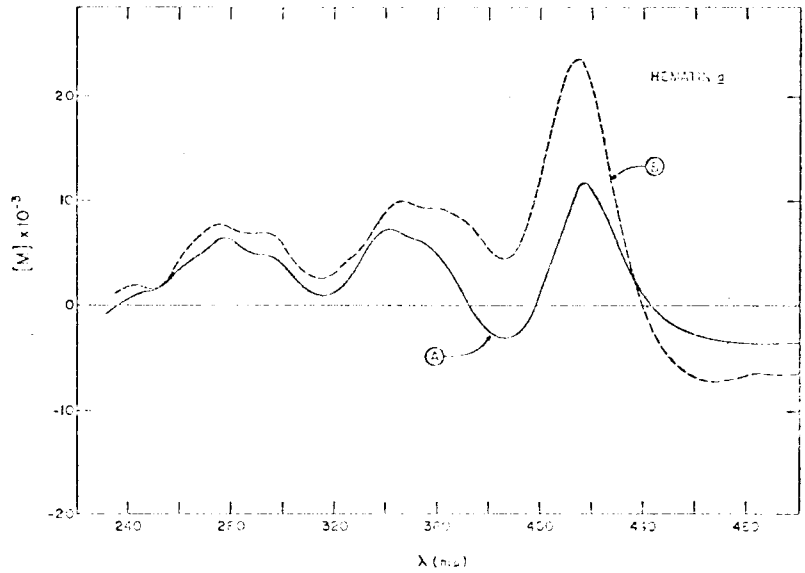

HEMATIN a
20
10
0
-10
-20
[M] x10⁻³
240
250
300
350
400
450
480
λ (mμ)
B
A

Fig. 5

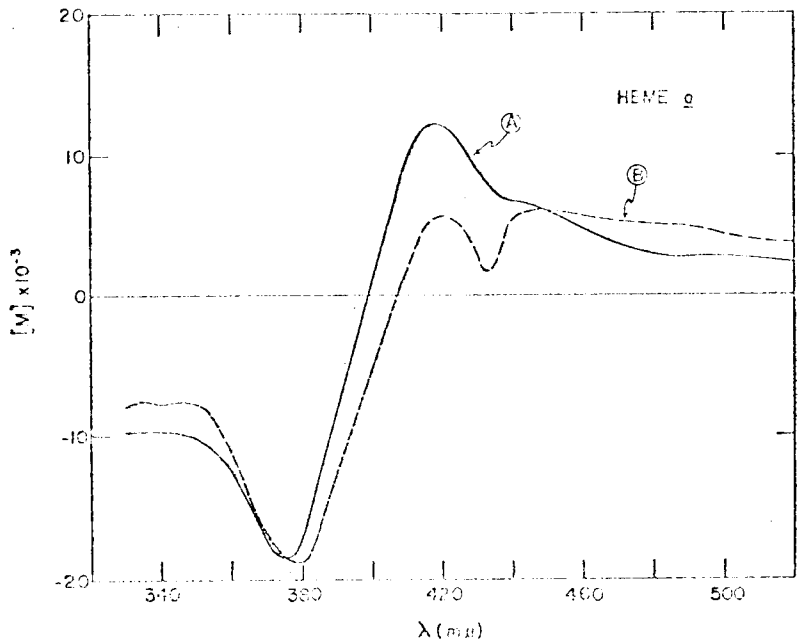

HEME a
20
10
0
-10
-20
[M] x10⁻³
340
380
420
460
500
λ (mμ)
A
B

Fig. 6

negative extremum was formed at 432.5 mμ. The negative extremum at 378 mμ remained unaffected. The resulting dispersion curve resembled that of Type II. The amplitudes of rotation (417.5 mμ - 378 mμ) were 24,500° and 31,000° in the presence and absence of NaCl respectively. The cross-over point was shifted from 398 mμ to 407.5 mμ upon addition of the salt. The absorption spectra in the Soret region were only slightly affected; the maximum was shifted from 406 mμ (without NaCl) to 409 mμ (with NaCl) and showed a small degree of hypochromicity.

The effect of sodium chloride on a concentrated solution of hematin a is summarized in Table III. The positive extremum at 417.5 mμ and the negative extremum at 387.5 mμ were affected in the same manner as in dilute solutions. However, the increase in rotation at the positive extremum was not so prominent. The amplitude of rotation (417.5 mμ - 387.5 mμ) which measured the effect of NaCl, gradually decreased as the salt concentration increased. The Soret absorption maximum was virtually unaffected and its extinction coefficient remained the same. The reduced solution of hematin a showed a decrease in rotation at 417.5 mμ (peak) but an increase in rotation at 375 mμ (trough) as the salt concentration was increased. Like the dilute solutions, both the positive and negative extrema were shifted towards longer wavelengths and the cross-over point was moved

TABLE III

Effect of NaCl on Type I Hematin a Concentrated Solution

Hematin a (μM)	NaCl (M)	Oxidized Soret Absorption maximum (mμ)	Oxidized ϵ mM^{-1} cm^{-1}	Oxidized ORD Amplitude* $[M] \times 10^{-3}$	Reduced Soret Absorption maximum (mμ)	Reduced ϵ mM^{-1} cm^{-1}	Reduced ORD Amplitude** $[M] \times 10^{-3}$	Reduced ORD Crossover point (mμ)
201	0	400	33.7	11.1	403	32.5	50	391
201	.05	400	33.6	9.0	403	32.8	45	392.5
201	.1	401	32.2	10.6	403	32.0	56	395
201	.2	401	32.8	7.5	403	33.0	54	401
201	.4	401	32.4	4.5	404	32.6	83	412

*Peak at 415 mμ - trough at 387 mμ in degree per mole of hematin a per liter per decimeter.

**Peak at 417 mμ - trough at 375 mμ.

from 391 mμ to 412 mμ. However, in contrast to dilute solutions, the negative extremum at 432.5 mμ (cf. Figure 6) was absent. The amplitude of rotation (417.5 mμ - 375 mμ) increased progressively from 50,000° (without salt) to 83,000° (with 0.4 M NaCl). A red shift of the Soret absorption maximum was also observed although the extinction coefficient remained fairly constant. A consistent phenomenon observed in concentrated but not in dilute solutions, was an initial decrease in the amplitude of rotation as soon as sodium chloride was added.

It is well known that chloride ions can act as ligands of hemes (71) and that salts induced aggregations (3, 37, 60). The observations described above probably reflect the action of chloride ions as ligands on the dispersion curves. However, this liganding effect becomes obliterated when high concentrations of sodium chloride are present because, at this stage, aggregation becomes a predominating factor that influences the rotatory dispersion curves. In dilute solutions, where chloride to heme ratio is larger, aggregation effect undoubtedly predominates.

Effect of Carbon Monoxide -- The ligand reaction of carbon monoxide on hematin a has been reported (34). The effect of CO on the ORD curves of hematin a is depicted in Figure 7 and the absorption spectrum in Figure 8. The CO-treated solutions of Types I and

Figure 7. ORD of carbon monoxide reacted heme a in 0.05 M phosphate buffer, pH 7.8. (A) Type I solution containing 198.5 μM hematin a, (B) Type II solution containing 221.4 μM hematin a. Light-path: 0.01 dm.

Figure 8. Absorption spectrum of carbon monoxide reacted heme a in 0.05 M phosphate buffer, pH 7.8. The system containing 198.5 μM hematin a, was reduced with sodium dithionite and bubbled with CO for 1 minutes. Light-path: 0.01 dm.

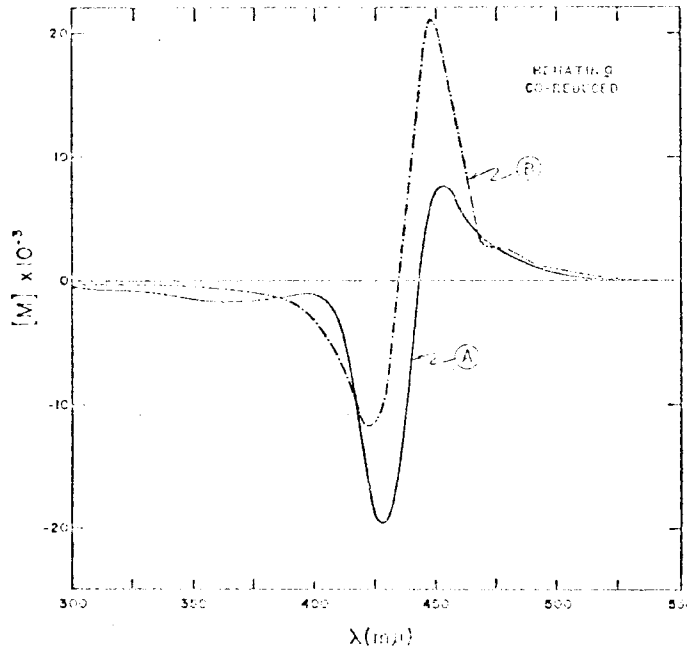


Fig. 7

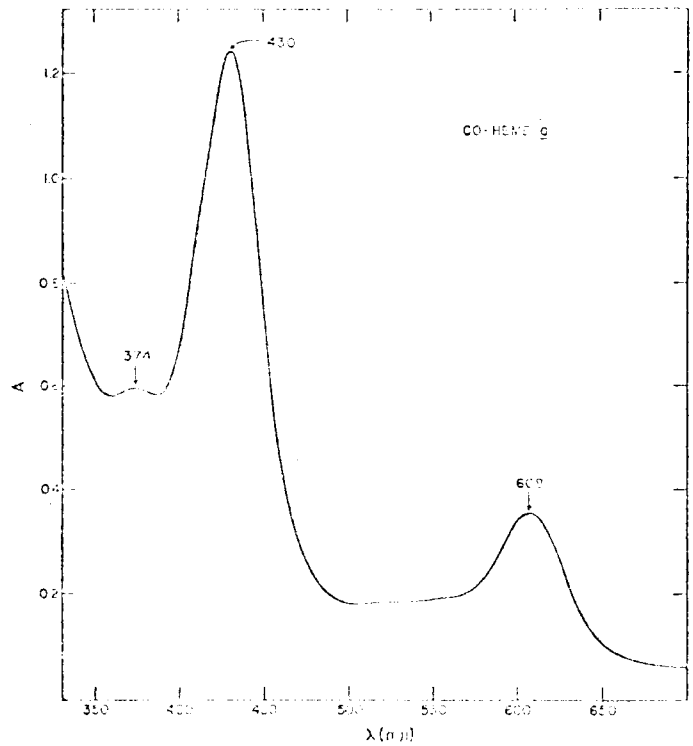


Fig. 8

II hematin a gave similar absorption spectrum with maxima at 608 and 430 mμ. However, the ORD curves were somewhat different. An apparent, relatively broad, unsymmetrical Cotton effect was observed having a peak at 454 mμ, a trough at 427.5 mμ, cross-over point at 442.5 mμ and an amplitude of 27,000° in Type I and 447.5 mμ (peak), 423 mμ (trough), 435 mμ (cross-over) and an amplitude of 32,000° in Type II. Neither inflection point coincided with the absorption maximum (430 mμ). This was somewhat expected since the ORD curves of both types were not composed of a simple Cotton effect but rather resembled that arising from a couplet.

Effect of pH -- The dispersion curves for Type I hematin a solution in both oxidized and reduced states were studied over a wide pH range. Figure 9 illustrates the ORD profile of hematin a as a function of pH. Under the conditions noted in the legend for Figure 9, increases in pH from 7.6 to 11.5 decreased the magnitude of rotation over the entire wavelength region. The rotation at 385-387.5 mμ, which was negative at neutral pH, became progressively more positive. For the pH range 8.5-11.5, there appear to be 3 isorotatory points corresponding respectively to wavelength at 431, 358 and 312 mμ. The Soret absorption maximum remained fairly constant in the region 400-402 mμ while the extinction coefficient decreased gradually as the pH was increased. Lowering the pH to 7.6 changed

Figure 9. ORD of hematin a as a function of pH.

The system contained 195-202 μM hematin a in 0.05 M phosphate. Curves: - - -, pH 5.9; ——, pH 7.6; — — —, pH 8.5; —·—·—, pH 9.5; —o—, pH 10.5 and —×—, pH 11.5. Light-path, 0.01 dm. Inset, molar rotation at 340 mμ as a function of pH.

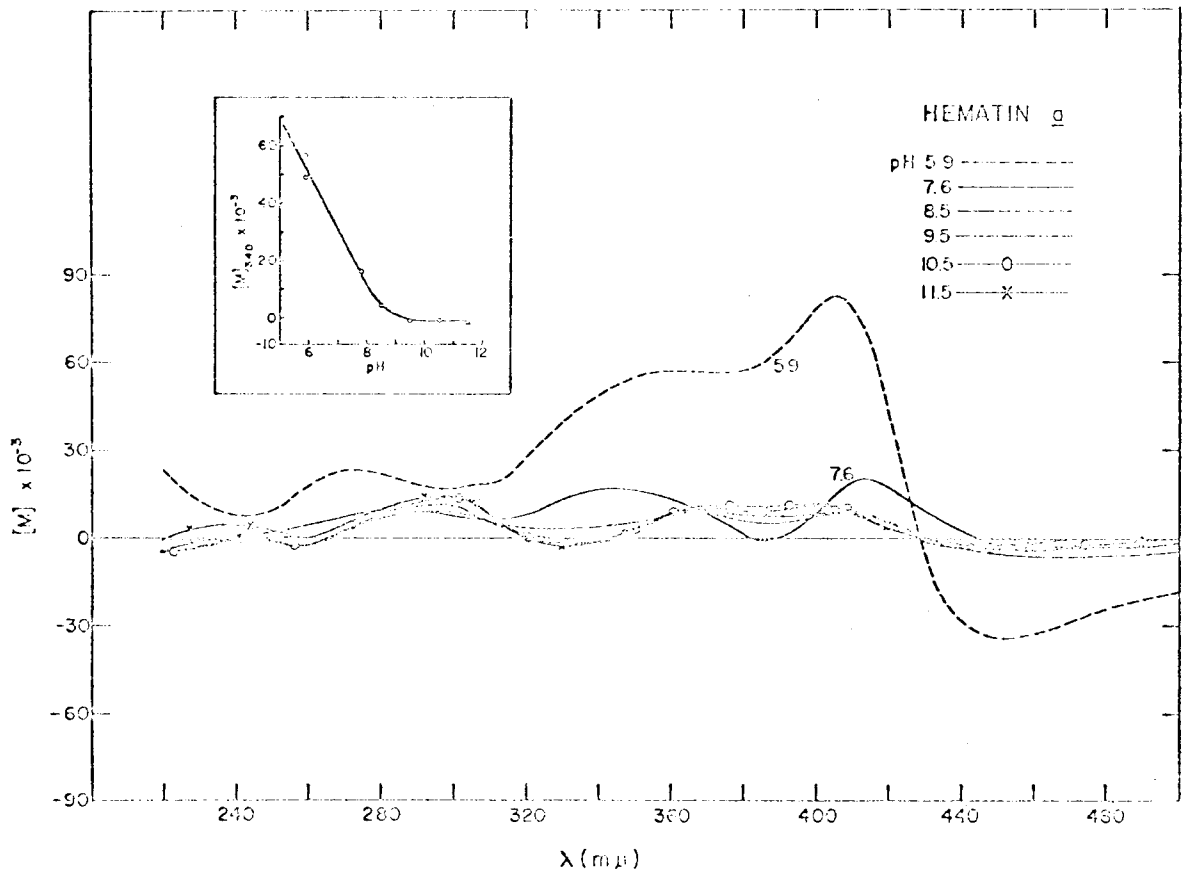


Fig. 9

the dispersion curve which differed slightly from that depicted in Figure 2. The variation was due to concentration and pH differences. However, the magnitude of rotation was quite similar. When the pH was adjusted to 5.9, the entire dispersion curve changed dramatically. It resembled that of Type II hematin a although the magnitude of rotation was not as large. There was a broad negative extremum at about 450 mμ followed by a positive extremum at 407 mμ. The amplitude of rotation was approximately 120,000°. Below pH 5, precipitation of the hemes gradually occurred; no reliable ORD data were obtained. These observations reflect that the increase in rotation and the change in the ORD profile in acidic medium are to a large extent the results of aggregation. A plot of the molar rotation at 340 mμ as a function of pH is given in the inset of Figure 9. The curve corresponds to one limb of a conventional titration curve. Precipitation of the heme prevents extension of the titration data to lower pH. Although the pK of the carboxylated groups on the heme cannot be accurately estimated from the plot, it must be lower than 6.5. Phillips (58) has reported the pK value for a dicarboxylated porphyrin (not hematin a) as 5.7. The presence of electrophilic groups such as formyl and vinyl in hematin a would undoubtedly affect the pK values.

Figure 10 illustrates the effect of pH on heme a (reduced hematin a). Increase in alkalinity over the range of pH 8.5-11.5 brought

Figure 10. ORD of heme a as a function of pH.

The system contained 195-202 μM hematin a in 0.05 M phosphate. Curves: ---, pH 5.9; ——, pH 7.6; — — —, pH 8.5; —·—, pH 9.5; —o—, pH 10.5; and —×—, pH 11.5. Inset, molar rotation at 373 mμ as a function of pH.

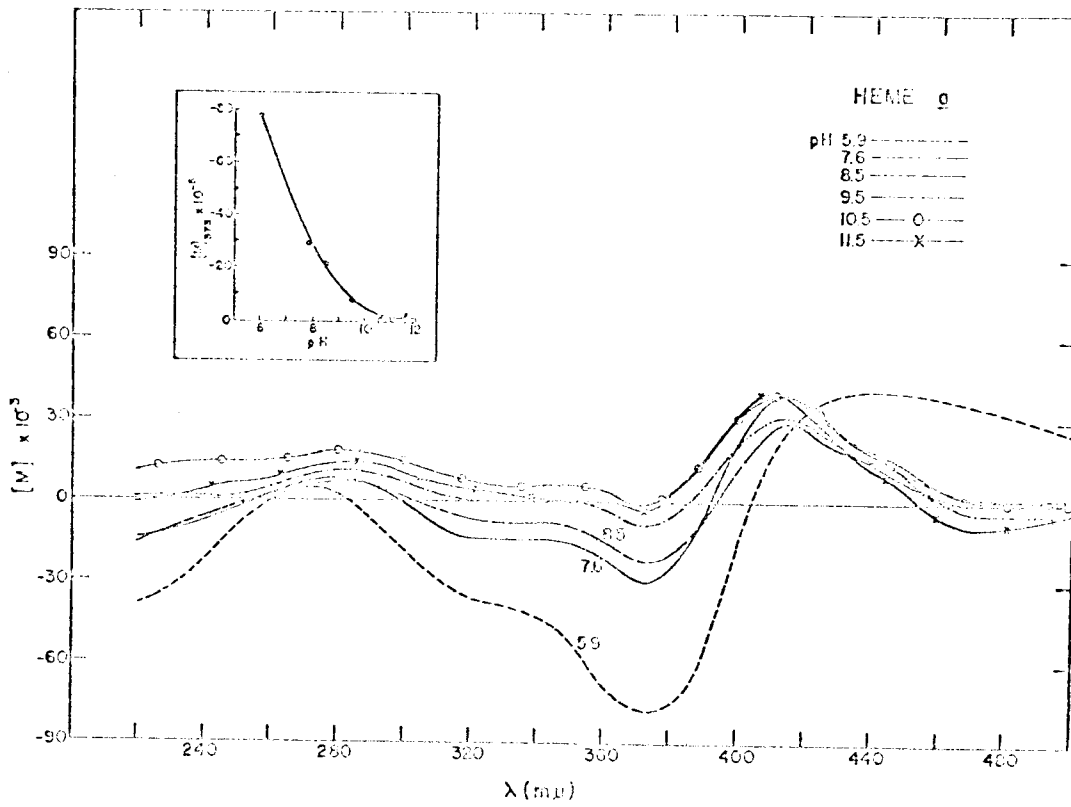

HEME g
pH 5.9
7.6
8.5
9.5
10.5
11.5
90
60
30
0
-30
-60
-90
[M] x 10³
240
280
320
360
400
440
480
λ (mμ)
8.5
7.6
5.9

Fig. 10

about a gradual displacement of the dispersion curves to more dextro-rotation. This is clearly shown when comparing the rotation at the trough (373-375 mμ). However, the shape of the dispersion curves remained practically the same. The amplitude of rotation varied between 40,000° and 50,000°. There was a gradual shift of the Soret absorption maximum towards longer wavelength as the pH was increased. The extinction coefficient of the spectra remained practically constant. In neutral medium (pH 7.5), the dispersion curve had a cross-over point at 392 mμ, a peak at 413 mμ and a trough at 373 mμ, with an amplitude of approximately 70,000°. In contrast, the rotations at pH 5.9 increased considerably over the entire wavelength region. The positive extremum was shifted to 443 mμ while the negative extremum remained at 373 mμ. The amplitude of rotation amounted to 120,000°. Again when the molar rotation at 373 mμ is plotted as a function of pH, as shown in the inset of Figure 10, the pK can be estimated as within the range 4.0-6.0.

The effect of pH on the dispersion curves of the carbon monoxide complex of heme a is shown in Figure 11. The change of the ORD profile with respect to pH paralleled those observed in the oxidized and reduced forms. In the region of pH 8.5-11.5, the dispersion curves showed a deep negative extremum at 430-431 mμ which corresponded to the absorption maximum of the solution. The rather

Figure 11. CRD of CO-reacted heme a as a function of pH. The system contained 195-202 μM hematin a in 0.05 M phosphate. Curves: ---, pH 5.5; ——, pH 7.6; — — —, pH 8.5; —.—, pH 9.5 and —o—, pH 11.5. Inset, molar rotation at 430 mμ as a function of pH.

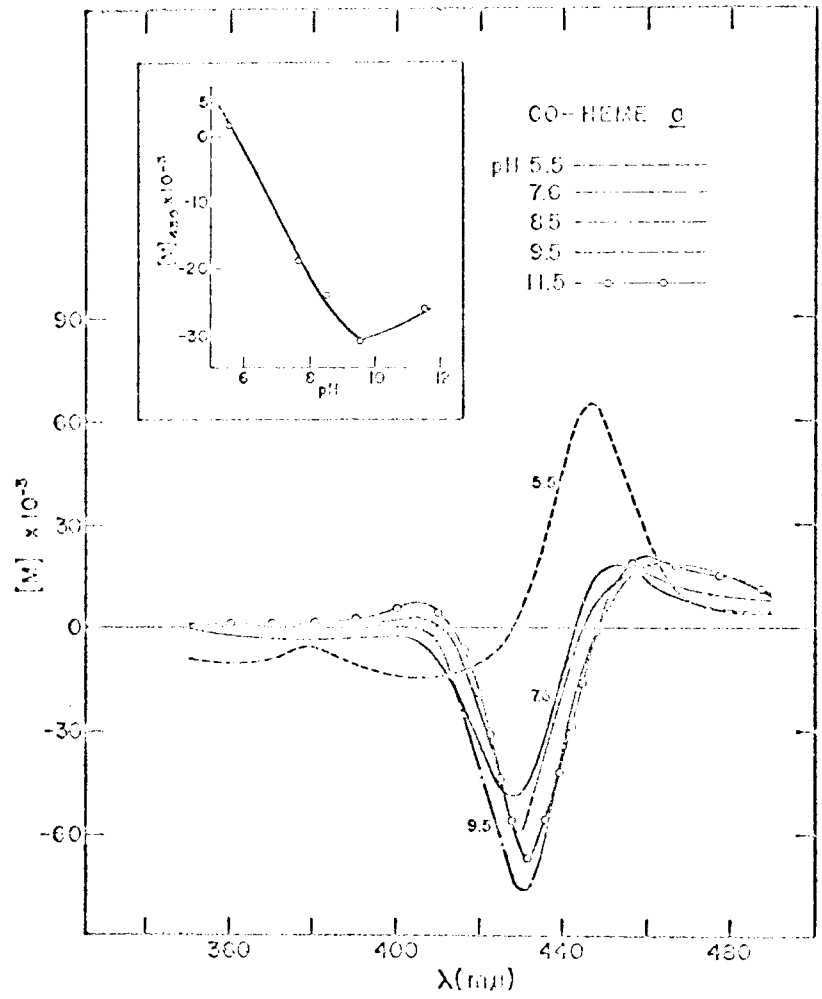

CO-HEME a
pH 5.5
7.0
8.5
9.5
11.5
5.5
7.0
9.5
90
60
30
0
-30
-60
360
400
440
480
λ(mμ)
[M] x10⁻⁵
6
8
10
12
pH

Fig. 11

symmetrical shape of the trough together with the inconspicuous peaks at both ends suggests that the observed rotation arises from a couplet with transitions originating very close to the absorption maximum and having approximately equal rotational strength but of opposite signs. The Soret absorption maximum remained constant (430-431 mμ); the extinction coefficient which gradually increased with pH did not exceed that at neutral medium.

However, the dispersion curve at pH 5.5 differed entirely from those at alkaline pH. There appeared a positive extremum at 447 mμ with no obvious negative extrema. The Soret absorption maximum was shifted to 437 mμ while the extinction coefficient was reduced to about one-half that at neutral pH. The hypochromicity and red-shift of the Soret absorption maximum suggest heme stacking in the process of aggregation in acidic medium. The observed rotatory behavior under these conditions may thus originate, at least to a great extent, from transitions due to the close proximity of the heme moieties. The dispersion curve at pH 7.5 corresponded to a type intermediate between the two forms just described. The profile was very similar to that described in Figure 7. A plot of molar rotation at 430 mμ against the pH values is given in the inset of Figure 11. The plotted curve is similar to those observed for the oxidized and reduced forms described earlier.

Effect of Solvent -- Practically no optical activity was observed when hematin a was dissolved in ether, dioxane, dimethyl sulfoxide, 2-chloroethanol, trichloroethanol, 50% aqueous ethanol, chloroform-pyridine (1:1), 20% aqueous pyridine, or 1% Emasol - 0.05 M phosphate buffer, pH 7.8. Although the absorption spectra of hematin a in 50% ethanol (cf. 78) and in Emasol-phosphate buffer (cf. 34) were similar to those in aqueous medium, certain differences were clearly discernible. Thus, in both cases, the Soret absorption maximum exhibited distinct hyperchromicity and was shifted to 434-436 mμ when reduced with dithionite.

ORD of Protohematin -- Solutions of protohematin were prepared by dissolving crystalline protohematin-HCl in the same manner as in hematin a solutions. No optical activity was observed in the oxidized, reduced and carbon monoxide-treated reduced forms at concentrations of 13.4 and 200 μM. Also, protohematin in the presence of 0.2 M sodium chloride showed no optical rotation.

Sedimentation Behavior[2] of Hematin a -- Ultracentrifuge studies on the sedimentation behavior of hematin a were conducted using various solvents. Table 4 summarizes the results.

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[2]The advice and assistance by Dr. R. Dyson and the useful discussion with Dr. G. Blauer are gratefully acknowledged.

TABLE IV

Sedimentation Coefficients of Hematin a in Various Solvents*

Hematin a solution	S_{20} (Svedberg Unit)
1. Type I**	8
2. Type II**	50***
3. Type I** + 0.2 M NaCl	12
4. at pH 11.1, 50 mM phosphate	10
5. in 50% ethanol, .02 M Tris	****
6. in 1% Emasol-50 mM phosphate buffer, pH 7.4****	****

*Hematin a concentrations were in the range 325-402 μM.

**See text for the experimental conditions.

***The sedimentation patterns were heterogenous; the value listed was for the main component.

****No sedimentation species was observed.

It may be noted that Type II hematin a solution shows a much higher aggregated form than Type I. Although the sedimentation pattern of Type I exhibited a comparatively broad peak, it appeared only as one species. Type II solution was heterogeneous and the value of 50 s belonged to the main component. In the presence of sodium

chloride or at pH 11.1, the sedimentation coefficient increased as compared to that of Type I. On the other hand, in 50% ethanol or in 1% Emasol-1130 - 0.05 M phosphate buffer, pH 7.5, hematin a solution did not show any sedimenting species under the conditions in which the ultracentrifugation was performed. Because of the limitation of the cell paths and the sensitivity of the instrument, the sedimentation behavior of hematin a solution could not be tested in as wide a concentration range as in the ORD studies.

DISCUSSION

From the results presented, it may be safely concluded that the observed optical activity of hematin a is derived from the properties of the molecule. No slightest indication of extraneous contaminations has been detected. In addition, optical activity is restored when iron is re-inserted into a pure sample of porphyrin a (78). The dependence of optical rotation upon its oxidation state further substantiates the conclusion. However, the origin of the optical activity of hematin a molecule is not so easily discerned. From the available evidence, it is likely that the observed optical rotatory dispersion properties are a composite of several contributions, perhaps mainly from aggregation, ligand formation, the stereochemistry of the porphyrin ring and side chains in the hematin a molecule, and the orderly structure or conformation of the molecule.

Aggregation and Optical Activity -- The effects of concentration, salt, and pH on the observed ORD behavior suggest that the molecules are in some form of aggregates. Protohematin has been known to form aggregates of rather high molecular weights in aqueous solution (37, 60) and probably in some form of micelles (3). In addition to these groups, such as methyl, vinyl and propionic acid which also exist in protohematin, hematin a has a formyl group at

position 8 and a large hydrophobic side chain at position 2. Thus it is not unreasonable to expect that hematin a may be more liable to form aggregates than protohematin. If there were a direct correlation between the magnitude of rotation of the dispersion curve and the degree of aggregation of hematin a, then the large increase in rotations for Type II hematin a solution would reflect a higher degree of aggregation. Indeed, ultracentrifuge studies fully support this view. A similar observation was made by Buchwald and Jencks (6) who observed a 30-fold increase in the maximal molar rotation following aggregation of astaxanthin. Thus, there seems to be a direct relation between the observed optical rotation and the size of aggregates.

It is noteworthy to mention that not all aggregated heme systems generate optical rotations. Protohematin, which has been shown to aggregate in the presence or absence of salt, shows no optical activities at all. The answer to the question as to why the aggregates of hematin a generate or at least augment optical rotations, may lie in the intrinsic structure of the molecule. As shown in Figure 1, the side chains are so arranged that porphyrin a itself is inherently asymmetric, but it might be expected that this asymmetry alone would cause optical activity in the far ultraviolet region probably beyond detection by the present instrumentation. However, when the iron is in the center of the plane as in heme a and the z-coordinate

of the iron is linked to different ligands above and below the porphyrin plane, the iron becomes the center of dissymmetry. By introduction of this new dissymmetry, the optical activity is enhanced and exhibits rotations in the visible region. This idea may be the case in the formation of hematin a aggregates. Nevertheless, this type of coordination aggregation (5) is not completely responsible for the observed optical rotations because the complex nature of the dispersion curves already reflects perturbations arising from heme-heme interactions as a result of close proximity of the heme planes as well as hydrophobic interactions of the long alkyl and other groups.

Heme-iron Linkages -- The observation that the ORD of hematin a is dependent upon its oxidation state also indicates that the heme-iron is involved in the formation of aggregates. A hydroxyl group and a water molecule (37) are known to coordinate the iron of protohematin in aqueous solution. The hydroxyl ligand may be replaced by a water molecule when the heme iron is reduced. If a similar situation also exists in aqueous hematin a solution at neutral pH, then we can rationalize a greater concentration effect on the ORD profiles of the reduced form than on those of the oxidized. In the oxidized form, the involvement of the hydroxyl ion in the linkage between hemes, in addition to the hydrophobic interactions of the long alkyl as well as other groups, would give a rigid structure of aggregates much less dependent on concentration. Such a linkage would be changed when

the hydroxyl ligand is displaced by a water molecule as in the case when the heme iron is reduced. This is precisely what we have observed.

Ligands and Organic Solvents -- The abolition of the anomalous rotations by pyridine or Emasol may be related to ligand formation and depolymerization of the aggregates. Formation of the dipyridyl compound would displace a solitary ligand from, say, position 5; this would either equalize the probabilities of two opposite nonplanar forms, or, assuming a planar hematin molecule and neglecting distant asymmetric configuration, be effectively symmetrical. Emasol-1130 is known to coordinate with the iron in free hematin a solution and,in turn even the absorption spectra of the hematin solution is changed (34, 78). This Emasol ligand might similarly be bound in positions 5 and 6 as in the case of the dipyridyl compound.

The disappearance of optical activity in organic solvents is probably due to the results of dissociation of the aggregates and destroying the orderly structure of hematin a which exists in aqueous neutral solutions. The conformation of the molecule may involve hydrophobic side chains as mentioned. In the presence of an organic solvent which possesses a lower dielectric constant, the hydrophobic groups of hematin a would interact more favorably with the solvent and thus the orderly structure is destroyed. The apparent dissociation of the aggregates by organic solvents is reflected from the ultracen-

trifuge studies as described.

Asymmetric Carbon Atoms and Optical Activity -- Porphyrin molecules of well-known structures such as the phäophorbid a and related compounds have been reported to exhibit both anomalous ORD and CD in dioxane (74). These rotatory properties are attributed to the asymmetric carbon atoms in the structures. Likewise, their counterpart, chlorophyll a, also possesses optical rotation in diethyl ether (22, 23). The ORD of the heme-octapeptide (derived from cytochrome c) in pure organic solvent, to our knowledge, has not been reported although it shows only a simple CD band in aqueous solution containing 1.18 M dioxane (64). In contrast, hematin a which possesses at least 3 asymmetric carbon atoms according to the structure proposed by Grassl, et al. (19, 20) or 1 asymmetric C-atom (C-1) according to structures proposed by Caughey, et al. (9), shows no optical activity in organic solvents. Thus all the proposed structures are incompatible with experimental observations. Lemberg (42) has suggested that the optical inactivity may be due to a keto-enol tautomerism between C-1 of side chain-2 and the formyl group of side chain-8. However such an equilibrium would hardly seem to favor the enol configuration of the carbonyl group on side chain-8, especially in organic solvents. The disparity perhaps lies in the true structural configurations of side chain-2 on the molecule. Indeed, there have been discrepancies between the studies by Morell,

et al. (47, 48) and Grassl, et al. (19, 20) who have proposed the structure for side chain-2 (cf. Figure 1), and the work by Caughey, et al. (9, 79) who believe that a hexoseamine group rather than a hydroxyl group is attached to C-1 and the alkyl group contains unsaturated bonds.

At any rate, it is difficult to assess the relative importance of (1) the asymmetric substituents on the porphyrin molecule, (2) the nonplanarity of the ring, and (3) coordinations at positions 5 and 6, in the contribution to optical activities. Two basic situations may be distinguished in which hematin a may be considered as an intrinsically dissymmetric chromophore or as a relatively symmetric chromophore but dissymmetrically perturbed. Under certain conditions the two are almost impossible to differentiate. These facets have been discussed at the recent Cytochrome Symposium in Kobe (78).

In summary, the precise assignment of the origin of optical activity in the molecule is not possible at present. Nonetheless, it is beyond any reasonable doubt that the ORD behavior of aqueous hematin a solution is indigenous to the molecule.

2. ANOMALOUS OPTICAL ROTATORY DISPERSION OF ALKALI-DENATURED CYTOCHROME c OXIDASE AND HEME-POLYLYSINE COMPLEXES

INTRODUCTION

Early in 1925, in one of his classical studies on the redox behaviors of cytochromes, Keilin (24) observed that strong alkali caused a blue shift of the α-band of reduced cytochromes from 602 mμ to 576 mμ in bee muscles and yeast cells. He attributed the effect to the modifications of cytochrome oxidase. These observations were later confirmed and extended further by various workers using purified cytochrome oxidases (40, 41, 57, 62). Lemberg (41) suggested that the observed spectral shift was due to Schiff-base formation involving an unprotonated ϵ-amino group of a lysyl residue from the protein moiety and the formyl group of hematin a. Working at about the same time as Lemberg (41), Takemori and King (62) obtained similar results and the explanation put forward by Lemberg was greatly strengthened by the finding (62) that the aldimine bond formed as a result of Schiff-base reaction could no longer be extracted with acid acetone. Later studies (31, 34) using polyamino acids as models of protein moiety, showed that polylysine in alkaline medium interacted with heme a through Schiff-base and ligand reactions, to form a red complex that gave similar

spectral properties as the alkali-denatured cytochrome oxidase. When the complex was hydrogenated with borohydride, the hemes likewise became fixed to the polypeptide and no longer acid acetone extractable. An important feature about the effect of alkali on cytochrome oxidase was the complete loss of enzymic activity following alkali treatment even in a brief time (less than 5 minutes) and subsequent neutralization. It was therefore suggested (41, 57) that the principle effect of alkali was to bring about a conformational change around the heme(s) environment, resulting that both Schiff-base and ligand coordination reactions with lysyl residues of the protein moiety could occur. This explanation is consistent with the report by Matsubara, et al. (46) who showed that cytochrome oxidase contains 39 lysyl residues. To further substantiate this postulate and to understand the mechanism of reaction thereof, we have chosen to study the optical rotatory dispersion behavior of alkali-denatured cytochrome oxidase as well as polylysine-heme a complex in the Soret absorption region. The results are interpreted in terms of conformational change and ligand heme-iron interactions. The origins of the optical rotatory power of heme a-polylysine complexes are also discussed.

EXPERIMENTAL PROCEDURES

Materials

Purified cytochrome oxidase and hematin a were prepared as described previously (34, 62). Poly-L-lysine HCl (M. W. 74, 000, $\bar{p}$ = 578). Poly-D-lysine HCl (Ave. mol. wt. 6, 000, Ave. $\bar{p}$ = 40) and a sample of hemin chloride (ferric chloride of protoporphyrin IX, 2x recrystallized) were procured from Mann's Research Laboratory. Determination of the concentration of cytochrome oxidase was made spectrophotometrically by using an extinction coefficient of 12 (mM^{-1} cm^{-1}) at 603 mμ for its reduced-oxidized spectrum (66). Concentrations of hematin a and protohematin were determined by the pyridine hemochromogen method (16). For the polyamino acids, their concentrations were determined gravimetrically and they were not corrected for moisture content.

Methods

Optical rotatory dispersion measurements were conducted with a Cary model 60 recording spectropolarimeter at 23° C as previously described (77). Absorption spectra were taken before and after the experiment in a Cary spectrophotometer model 14 or 11. No changes of the absorption spectra were observed. Measurements of pH were made with a Beckmann Zeromatic pH meter.

Rotations are expressed as molar rotations in the unit of degrees per mole hematin a per liter per dm and they are not corrected for refractive index dispersion.

For the determination of the binding constant of the system, heme a-poly-L-lysine, the complex formation is considered as an equilibrium involving one heme a molecule (H) and a lysyl residue (L) of polylysine.

$$H + L \rightleftharpoons HL \tag{1}$$

The equilibrium constant is given by

$$K_c = \frac{[HL]}{[H][L]} \tag{2}$$

If C_H is the initial concentration of heme a; C_L, the initial concentration of polylysine and C_c, the concentration of the complex at equilibrium, then

$$K_c = \frac{C_c}{[C_H - C_c][C_L - C_c]} \tag{3}$$

Since polylysine does not absorb in the Soret wavelength region (428 mμ) as the complex, the total absorbance (A) at 428 mμ for the system can be written as:

$$A = \epsilon_c C_c d + \epsilon_H [H] d \tag{4}$$

where ϵ_c is the molar absorbance of the complex; ϵ_H, is the molar absorbance of heme a; $[H]$, the concentration of uncomplexed

heme a and d, the light path. Assuming that one complex is formed from one molecule of heme a, ,

$$C_H = C_c + [H] \tag{5}$$

Eqn. (4) can be transformed as,

$$A = \epsilon_c \cdot C_c \cdot d + \epsilon_H \cdot (C_H - C_c) \cdot d$$

$$= \epsilon_H \cdot C_H \cdot d + C_c \cdot (\epsilon_c - \epsilon_H) \cdot d \tag{6}$$

Let A_o be the absorbance of initial concentration of heme a,

$$A_o = \epsilon_H C_H \cdot d \tag{7}$$

then Eqn. (6) becomes,

$$A = A_o + C_c (\epsilon_c - \epsilon_H) \cdot d \tag{8}$$

Rearranging,

$$C_c \cdot d = \frac{A - A_o}{\epsilon_c - \epsilon_H} \tag{9}$$

Eqn. (3) can be written as,

$$\frac{1}{K_c} = (C_H - C_c)\left(\frac{C_L}{C_c} - 1\right) \tag{10}$$

If polylysine is in great excess i.e. $\frac{C_L}{C_c} \gg 1$ in Eqn. (10), then

$$\frac{1}{K_c} = (C_H - C_c)\frac{C_L}{C_c} \tag{11}$$

$$= \frac{C_L \cdot C_H}{C_c} - C_L \tag{12}$$

Replacing C_c by Eqn. (9) gives

$$\frac{1}{K_c} = \frac{C_L \cdot C_H (\epsilon_c - \epsilon_H) d}{A - A_o} - C_L \tag{13}$$

Rearranging,

$$\frac{C_L \cdot C_H}{A - A_o} = \frac{1}{K_c(\epsilon_c - \epsilon_H) \cdot d} + \frac{C_L}{(\epsilon_c - \epsilon_H)\, d} \qquad (14)$$

Thus, by titrating a fixed concentration of heme a with varying amount of polylysine, the results can be computed and plotted as the function $C_H \cdot C_L / A - A_o$ against C_L. From the intercept and slope of the straight line, the binding constant can be computed.

Spectropolarimetric titrations of either heme a-poly-L-lysine or protoheme-poly-L-lysine systems were performed by keeping the heme concentration constant and varying the amount of poly-L-lysine. A fresh solution was used for each concentration of poly-L-lysine. The solution was reduced with a small amount of sodium dithionite for 10 minutes before measurement in the polarimeter.

Nomenclature -- The terms hematin a and protohematin refer to the oxidized state; heme a and protoheme to the reduced state. When in general description where the oxidation state is not a point of significance, hematin and heme are used interchangeably.

RESULTS

ORD of Alkali Denatured Cytochrome c Oxidase -- As shown in Figures 12 and 13, alkali affects both the absorption spectra and the ORD behavior of the enzyme. Referring only to the Soret absorption maximum, the enzyme which had been incubated at pH 11.6 under conditions (62) as detailed in the legend of Figure 12 showed absorption maxima at 408 mμ (oxidized), 428 mμ (reduced) and 424 mμ (CO-reduced). The ORD profile as depicted in Figure 13, likewise showed marked changes. The oxidized form of the denatured enzyme exhibited practically no anomalous rotation in this region. Upon reduction by dithionite, a negative Cotton effect with the negative extremum at 437.5 mμ and the positive extremum at 427.5 mμ was observed. However, the observed profile was by no means simple. A small positive extremum at about 455 mμ was evident. The observed anomalous rotation in this region most probably originated from at least two Cotton effects superimposed on the protein backbone rotation. The negative Cotton effect, centered at about 430 mμ (cf. Figure 12), appears to possess greater rotational strength and therefore contributes more to the observed anomalous rotation in this region. Carbon monoxide did not alter the ORD profile of the reduced enzyme but shifted the anomalous

Figure 12. Absorption spectra of alkali-denatured cytochrome c oxidase.

The system containing 15 μM cytochrome c oxidase in 0.01 M phosphate, was adjusted to pH 11.6 and incubated at room temperature for 3 hours. The solution was scanned as such (oxidized), reduced with dithionite (reduced) and subsequently bubbled with carbon monoxide for 1 minute (reduced-CO).

Figure 13. ORD of the oxidized, reduced and CO-reacted alkali-denatured cytochrome c oxidase.

The system containing 20.85 μM cytochrome oxidase, was incubated in 0.01 M phosphate, pH 11.6 for 3 hours at room temperature.

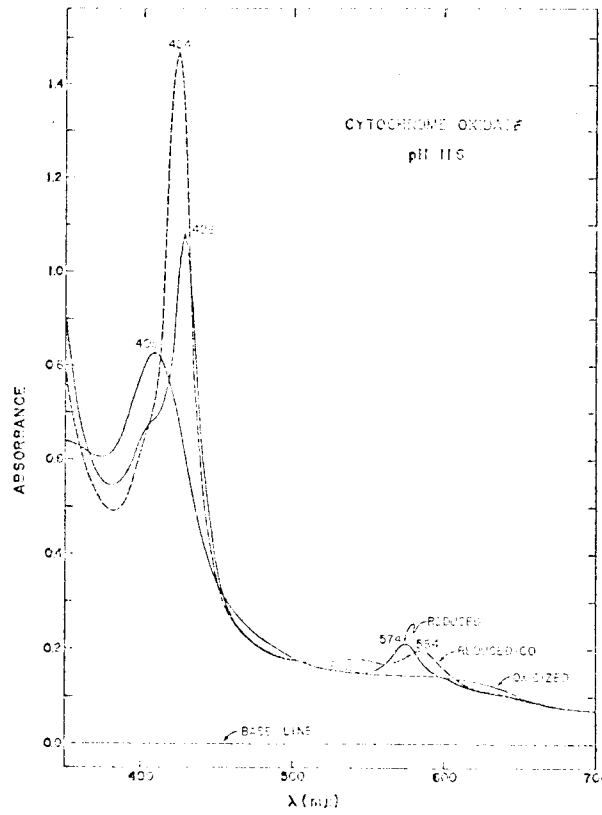
CYTOCHROME OXIDASE
pH 11.5
ABSORBANCE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
BASE LINE
400
500
600
700
λ (mμ)

Fig. 12

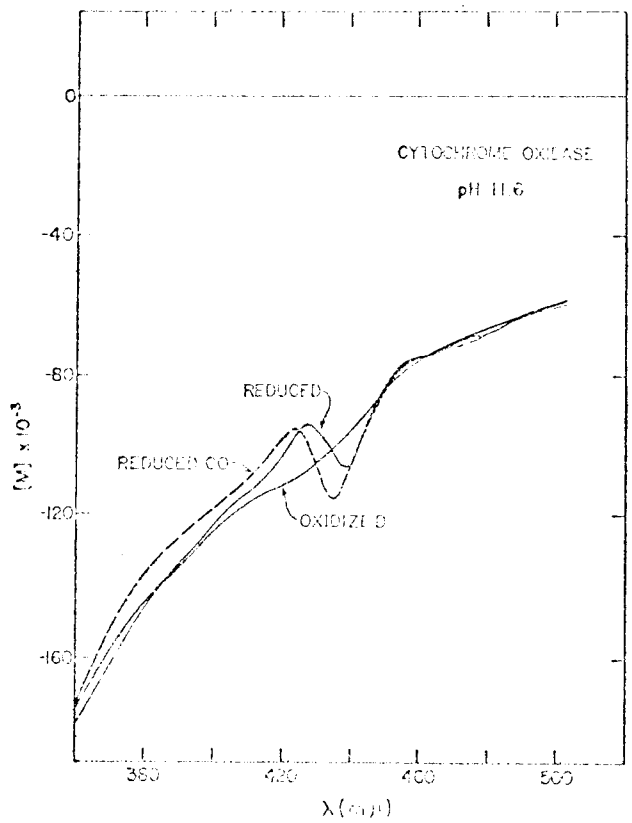
CYTOCHROME OXIDASE
pH 11.6
0
-40
-80
-120
-160
REDUCED
REDUCED CO
OXIDIZED
[M] x 10⁻³
380
420
460
500
λ (mμ)

Fig. 13

region to shorter wavelength. Thus the negative extremum which showed increased levo-rotation, appeared at 435 mμ while the positive extremum at 425 mμ. The small positive extremum (455 mμ) remained unchanged. Again the main Cotton effect appeared to center close to the absorption maximum of the CO-reduced form (424 mμ). The magnitude of rotation (peak-trough) was about 15,000° for the reduced and increased to about 20,000° for the CO-treated. A prominent feature of the alkali effect was the significant increase in levo-rotation of the entire dispersion curve. This is typical for protein denaturation. The observed ORD behavior of the alkali denatured enzyme was in marked contrasts to those exhibited by the native enzyme in neutral medium (35, 65). In fact, it was also observed that increasing the basicity of the solution, for example to pH 13, abolished completely the anomalous rotation observed for both the reduced and the CO-treated enzyme at pH 11.6.

ORD of Heme a-Poly-L-lysine Complex -- The interactions of hematin a with poly-L-lysine in alkali medium and its spectral characteristics in different states had been reported (34). The absorption spectra of the complex are given in Figure 14. Under the conditions of the reaction, an excess amount of poly-L-lysine was used so that all the hemes would be bound to the lysyl residues. The oxidized complex which had a Soret absorption maximum at 400 mμ, exhibited no anomalous rotations in this region. However, upon reduction of

the complex, a positive Cotton effect showing a sharp positive extremum at 435 mμ and a moderately broad negative extremum at 422.5 mμ was observed. The dispersion curve does not correspond to a simple Cotton effect but rather consists of a main positive Cotton effect originating close to the absorption maximum of the complex (428 mμ) and a small negative Cotton effect occurring at longer wavelength (cf. Figure 15). When the complex was treated with CO, the induced Cotton effect was shifted towards shorter wavelength. The two extrema became more symmetrical with a cross-over point close to the absorption peak of the complex (424 mμ). The amplitude was about 113,000° for the reduced and 128,000° for the CO-reduced forms. No induced Cotton effect has been detected in the long wavelength region corresponding to the α and β absorption maxima of the complex in all the three different states (oxidized, reduced, and CO-treated). It is interesting to note that although the spectral properties of the complex and the alkali-denatured cytochrome oxidase were very similar, their ORD behavior appeared to be considerably different. The distinct trough observed for the reduced enzyme (cf. Figure 13) was absent in the poly-L-lysine-heme a complex, which, on the contrary, showed a distinct positive peak. It appeared that the ORD profiles of the complex, either reduced or CO-treated, were the opposite of those exhibited by the denatured enzyme. Furthermore,

Figure 14. Absorption spectra of oxidized, reduced and CO-reacted heme a-poly-L-lysine complex in 0.05 M phosphate, pH 11.6. The solution contained 13.8 μM hematin a and 400 mμM poly-L-lysine (1.8×10^{-4} M, monomer unit). The complex was reduced by dithionite and subsequently reacted with carbon monoxide for 1 minute.

Figure 15. ORD of reduced and CO-treated heme a-poly-L-lysine in 0.05 M phosphate, pH 11.6.
The system containing 13.8 μM hematin a and 400 mμM poly-L-lysine (monomer concentration 1.8×10^{-4} M) was reduced by dithionite and subsequently bubbled with carbon monoxide for 1 minute.

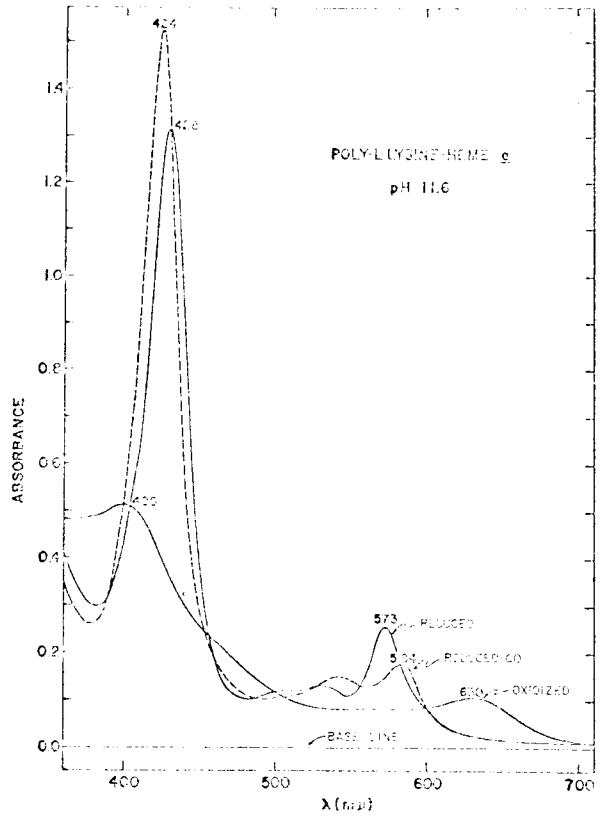
POLY-L-LYSINE-HEME c
pH 11.0
ABSORBANCE
λ (mμ)
REDUCED
REDUCED-CO
OXIDIZED
BASE LINE

Fig. 14

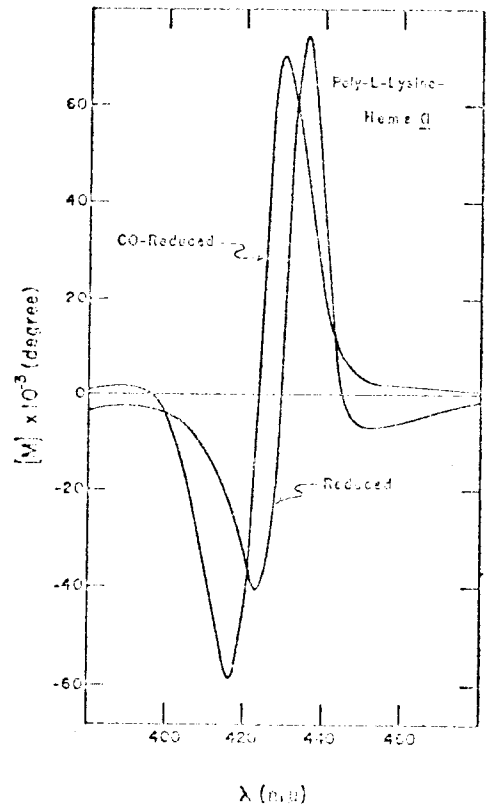
Poly-L-Lysine-
Heme c
CO-Reduced
Reduced
[M] x 10⁻³ (degree)
λ (mμ)

Fig. 15

the magnitude of rotation of the complex was several fold greater than those of the denatured enzyme. These observations were actually expected because poly-L-lysine in the media at pH higher than 11.6 is completely in the α-helical structure in contrast to the protein moiety of cytochrome oxidase which, in alkaline medium, is certainly denatured to a significant extent.

ORD of Heme a-Poly-D-lysine Complex -- When excess amount of poly-D-lysine was mixed with hematin a under conditions similar to the formation of poly-L-lysine-heme a complex, it was found necessary to increase the pH of the medium to 12.0 in order to bring about significant complex formation. The absorption spectra of the complex were identical to those of the L-forms, as depicted in Figure 14. However the ORD profiles appeared to be different. In Figure 16 are shown the results of rotation measurement over the Soret region for the reduced and CO-treated poly-D-lysine-heme a complex. The reduced form showed a broad positive extremum at 440 mμ followed by a sharp negative extremum at 427.5 mμ. Carbon monoxide changed the dispersion profile of the complex. The positive extremum at 440 mμ broadened and decreased in rotation while a new positive extremum emerged at 412.5 mμ. The negative extremum was shifted to 425 mμ, becoming sharp and narrow. The characteristics of the dispersion curves, having a dominant trough flanked by two small peaks, is typical of a couplet which, in this

Figure 16. ORD of reduced and CO-reacted heme a-poly-D-lysine in 0.05 M phosphate, pH 12.0.

The solution containing 13.5 μM hematin a and 8.33 μM poly-D-lysine (3×10^{-4} M, monomer) was reduced with dithionite and subsequently bubbled with carbon monoxide for 1 minute.

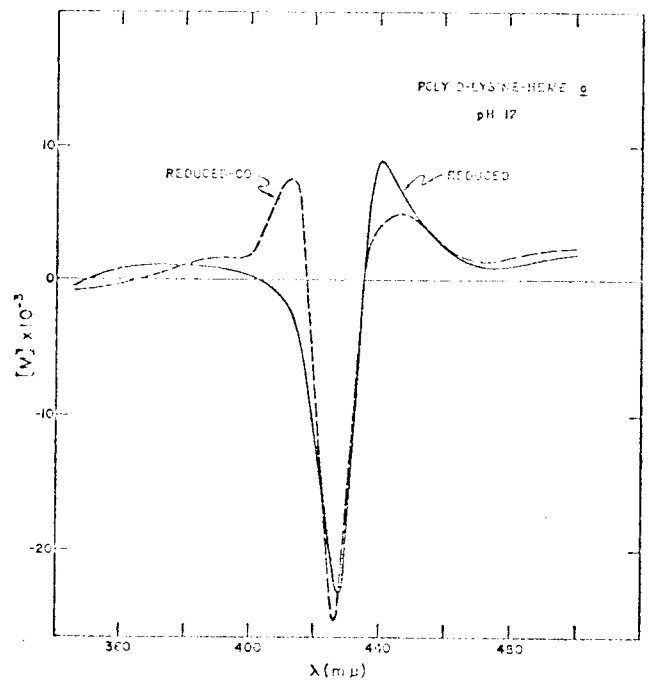
POLY D-LYSINE-HEME 9
pH 12
REDUCED-CO
REDUCED
10
0
-10
-20
[M]λ x10⁻³
360
400
440
480
λ(mμ)

Fig. 16

case, involves at least two optically active transitions of approximately equal rotational strength but opposite in sign, originating close to the absorption maximum of the complex (424 mμ). Like the alkali-denatured oxidase and the poly-L-lysine-heme a, the oxidized form of the complex showed no anomalous rotation in the Soret region. Likewise, no Cotton effect was observed for the α and β absorption maxima corresponding to either the reduced or the CO-treated states. A striking feature of these dispersion curves, is, however, their similarity at least qualitatively, to those exhibited by the denatured enzyme.

In Figure 17 are shown the protocols of the ORD of poly-L-lysine at pH 11.6 and poly-D-lysine at pH 12.0. It is obvious that the dispersion curve of one is the mirror image of the other, as depicted by the L or D configuration. The rotations for both forms at wavelength above 300 mμ are negligible at moderate concentrations. The magnitude of rotation at 233 mμ for poly-L-lysine is less than that corresponding to 100% helix, as reported in the literature (13). This is because the experimental results have not been corrected for moisture content which is about 10-12%.

Determination of the Binding Constant for Heme a-Poly-L-lysine Complex -- The results of titrating a fixed concentration of heme a with varying amount of poly-L-lysine were computed accord-

ing to the formulation derived above (cf. this section on Methods). The plot of $C_H \cdot C_L$ / A-A_o against C_L is illustrated in Figure 18. The plot is linear at high concentrations of poly-L-lysine but gradually deviates from linearity as its concentration decreases. The binding constant (the initial step of binding one molecule of heme a to one lysyl residue of the polypeptide) calculated from the plot for both reduced and CO-treated forms varied within the range 8.5×10^6-1.7×10^7 M^{-1}. Values within the same range were also obtained by a similar treatment of the α-band of both reduced and CO-treated forms. The value of the binding constant which is in the order of 10^7, indicates the very high affinity of the unprotonated ϵ-amino group of lysyl residue for the heme iron. Indeed, concentration of poly-L-lysine as low as 0.03 mg per ml has been shown to form the red complex with heme a (34). The process is therefore thermodynamically favorable.

Spectropolarimetric Titration of Heme a-Poly-L-lysine System in the Absence of Emasol-1130 -- The results of measuring the changes in the ORD of poly-L-lysine-heme a as a function of poly-lysine concentration or the monomeric lysyl residues to heme a ratio (L/H), are shown in Figure 19 for the reduced and Figure 20 for the CO-treated forms. Free heme a has been shown to exhibit optical rotation under these conditions (Section 1, this thesis). It showed a positive extremum at 415 mμ which gradually decreased with

Figure 17. ORD of poly-L-lysine and poly-D-lysine in the UV-region.

The polypeptide was dissolved in 0.05 M phosphate at pH 11.6 for poly-L-lysine and at pH 12.0 for poly-D-lysine. The residue rotation was not corrected for refractive index dispersion.

Figure 18. A plot of the function of $C_L \cdot C_H / A-A_o$ and the concentration of poly-L-lysine (C_L) in heme *a*-poly-L-lysine complexes.

Curve (A), reduced form, absorbance (A) measured at 428 mμ.

Curve (B), CO-treated, reduced form, absorbance (A) measured at 424 mμ. C_H and A, concentration and absorbance of hematin *a*, respectively.

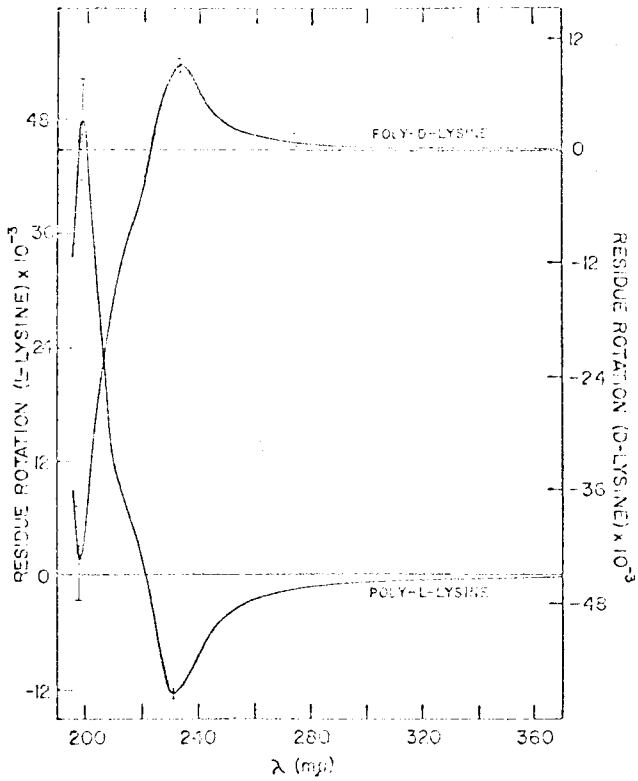
POLY-D-LYSINE
POLY-L-LYSINE
RESIDUE ROTATION (L-LYSINE) x 10⁻³
RESIDUE ROTATION (D-LYSINE) x 10⁻³
λ (mμ)
48
36
24
12
0
-12
-24
-36
-48
200
240
280
320
360

Fig. 17

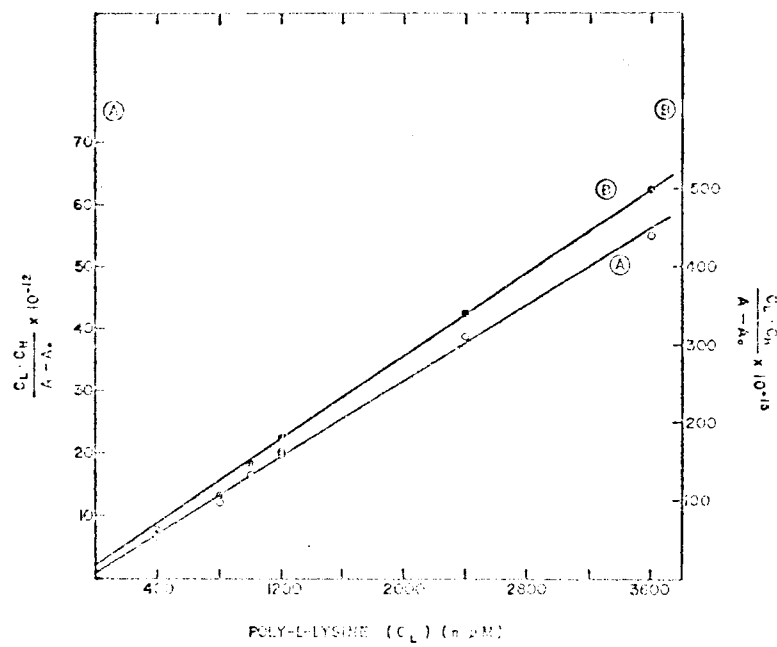
Ⓐ
Ⓑ
70
60
50
40
30
20
10
500
400
300
200
100
1200
2800
3600
POLY-L-LYSINE

Fig. 18

Figure 19. Spectropolarimetric titration of heme a-poly-L-lysine as a function of poly-L-lysine concentrations. Buffer medium: 0.05 M phosphate, pH 11.6 (No Emasol). Titration performed by mixing 13.8 μM hematin a with different amounts of poly-L-lysine and the solution was reduced with dithionite for 10 minutes prior to ORD measurements. L/H: number of lysyl residues per heme molecule.

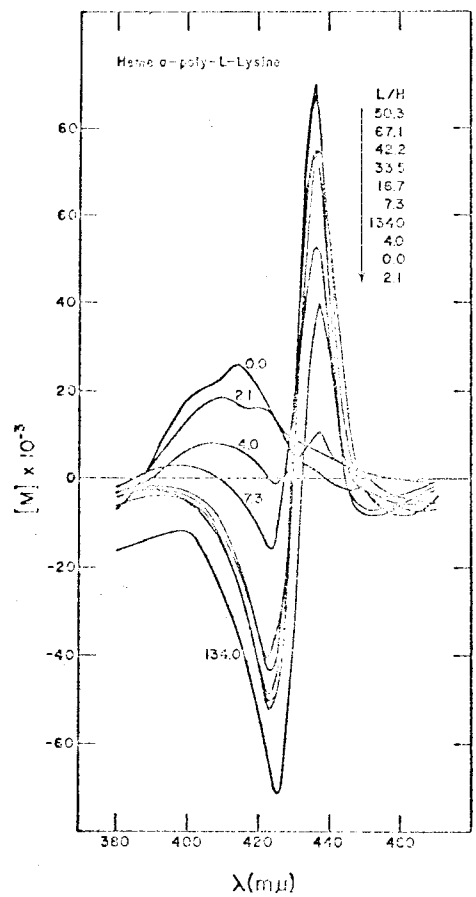
Heme a-poly-L-Lysine
L/H
50.3
67.1
42.2
33.5
16.7
7.3
134.0
4.0
0.0
2.1
60
40
20
0
-20
-40
-60
0.0
2.1
4.0
7.3
134.0
[M] x 10⁻³
380
400
420
440
460
λ(mμ)

Fig. 19

increase concentrations of polylysine. At L/H ratio of 7.3, the previously observed positive extremum for free heme a was completely abolished. The dispersion curve then resembled that of heme a-poly-L-lysine complex (cf. Figure 15), having a maximum at 435 mμ and a minimum at 422.5 mμ. The apparent inflection point was near 429 mμ. The magnitude of rotation at the peak was about three times greater than that at the trough. Addition of more polylysine increased the rotation at both extrema. However, the increment reached a maximum at L/H ratio of 50.3. Further addition of polylysine decreased the positive extremum. When the L/H ratio reached 134, the positive extremum was reduced to about half its maximum value while the rotation at the negative extremum increased considerably. The entire dispersion curve appeared to be displaced levo-rotatory. This is due to the peptide rotation which become significant at high concentration of poly-peptide (3200 mμM or L/H = 134). If the amplitude of rotation (peak-trough) was plotted as a function of polylysine concentration, the curve as shown in Figure 21, likewise indicates a decrease in the amplitude of rotation after passing through the maximum. This is in marked contrast to the spectrophotometric titration reported previously where the Δ absorbance value remaincd constant even with further increase in polylysine concentration (34). The magnitude of rotation at the maximum, corresponding to the L/H ratio of 50.3 was

Figure 20. Spectropolarimetric titration of CO-heme a-poly-L-lysine as a function of poly-L-lysine concentrations. Buffer medium: 0.05 M phosphate, pH 11.6 (No Emasol). The system contained 13.8 μM hematin a and various amounts of poly-L-lysine. The solution after dithionite reduction was bubbled with carbon monoxide for 1 minute before ORD measurements.

L/H: number of lysyl residues per heme molecule.

Figure 21. Titration curves of the systems heme a-poly-L-lysine and CO-heme a-poly-L-lysine (No Emasol). Amplitudes are peak-trough values obtained from curves shown in Figures 19 and 20.

Reduced: heme a-poly-L-lysine.

Reduced -CO: carbon monoxide treated heme a-poly-L-lysine.

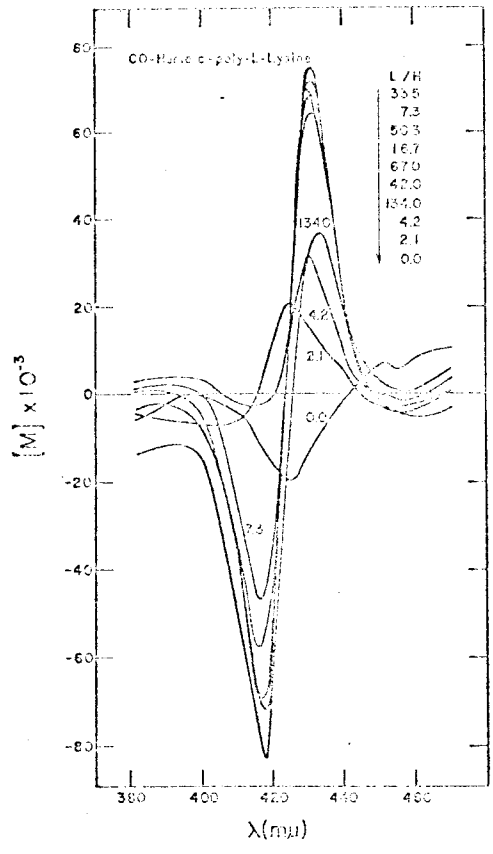
CO-Horse c-poly-L-Lysine
L/R
335
73
50.3
16.7
67.0
42.0
134.0
4.2
2.1
0.0
134.0
4.2
2.1
0.0
7.3
[M] x10⁻³
λ(mμ)

Fig. 20

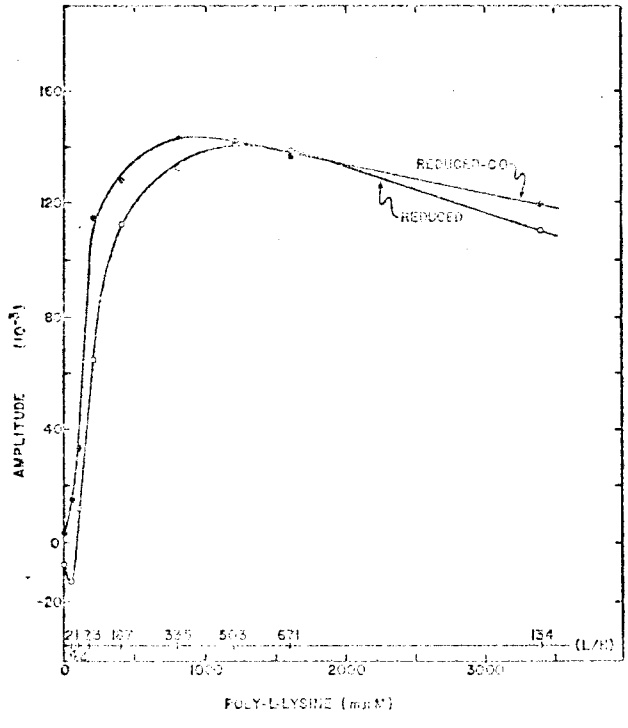
REDUCED-CO
REDUCED
AMPLITUDE
134
(L/R)
POLY-L-LYSINE (mM)

Fig. 21

143,000°. Because of the decrease in rotation at higher polylysine concentration, the lysyl residues per heme a molecule at saturation, cannot be estimated from the titration curve.

For the CO-treated heme a-polylysine complex, the ORD curves measured as a function of polylysine concentrations, were shown in Figure 20. Free CO-heme a in absence of polylysine, exhibited a negative extremum at 425 mμ. This extremum was completely abolished with 50 mμM polylysine (L/H = 2.1) and gave, instead, a positive extremum at 425 mμ. This peak was shifted to 430 mμ at higher concentration of polylysine (L/H = 4.3). When the ratio reached 7.3, the dispersion curve changed to a simple Cotton effect having extrema at 430 mμ (positive) and 416 mμ (negative). The inflection point was 423 mμ, close to the absorption maximum of the complex. The amplitude at maximum value corresponding to the L/H ratio of 33.5, was 143,000°. Further increase in the concentration of polylysine gradually decreased the amplitude. At very high concentration of polylysine (3200 mμM, L/H = 134), the entire dispersion curve was displaced levo-rotatory due to the peptide backbone rotation. When the amplitude of rotation (peak-trough) was plotted as a function of polylysine concentration, the curve as shown in Figure 21, behaved in a similar manner as the reduced form. There was a rapid rise in the amplitude value at low concentration of polylysine. It levelled off to a maximum

value when the L/H ratio reached 33.5. Beyond this value, the amplitude gradually decreased again although not as rapid as the reduced form.

Spectropolarimetric Titration of Heme a-Poly-L-lysine System in Presence of Emasol-1130 -- It is known that heme a in presence of a small amount of Emasol-1130, does not possess any optical activity (77). Sedimentation experiments (cf. Section 1) indicated that the hemes were monomeric dispersed under these conditions. Thus the formation of the red complex in presence of Emasol-1130, was studied as a function of poly-L-lysine concentration. The absorption spectra of the red complex formed under these conditions were similar to those depicted in Figure 14 for the oxidized, reduced and CO-treated forms. However, the ORD behavior appeared to be quite different. Figures 22 and 23 illustrate the results of ORD measurements for the reduced and the CO-treated forms of the complex at various polylysine concentrations. In contrasts to earlier observations (cf. Figures 19 and 20), the reduced complex, formed at polylysine concentration 175 mμM (L/H ratio = 6.8) showed a dominant peak at 436 mμ flanked by two smaller troughs at 455 mμ and 425 mμ respectively. The shape of the dispersion curve is typical of those originating from coupled electronic transitions. Further addition of polylysine to 200 mμM (L/H = 7.8) increased the rotation at the negative as well as the

Figure 22. Spectropolarimetric titration of heme a-poly-L-lysine as a function of poly-L-lysine concentrations. Buffer medium: 0.05 M phosphate containing 1% Emasol-1130, pH 11.6. Titration performed by mixing 14.9 μM hematin a with different amounts of poly-L-lysine and the solution was reduced with dithionite for 10 minutes prior to ORD measurements.

L/H: number of lysyl residues per heme molecule.

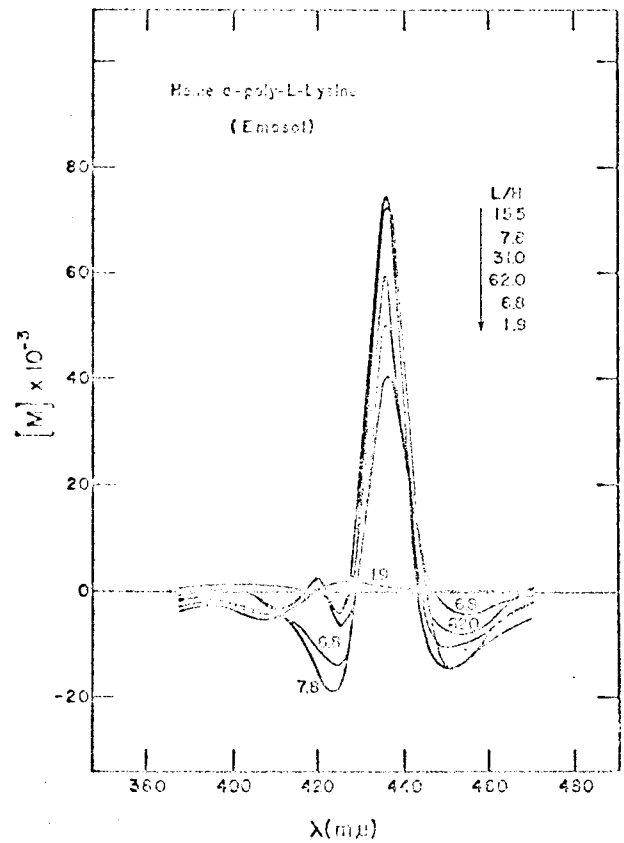
Mouse α-poly-L-Lysine
(Emosol)
L/H
15.5
7.8
3.0
62.0
6.8
1.9
80
60
40
20
0
-20
[M] x 10⁻³
360
400
420
440
460
480
λ(mμ)
1.9
6.8
62.0
3.0
15.5
7.8

Fig. 22

positive extrema. When the polylysine concentration reached 400 mμM and higher, the rotation at the positive extremum remained constant and then decreased while a new positive extremum of rather small magnitude of rotation became evident at 420 mμ. The dispersion curve which corresponded to a couplet at polylysine concentration 175-200 mμM, became complex and consisted of at least more than two Cotton effects when the polylysine concentrations were increased to 400 mμM or higher. If the amplitude of rotation (peak at 436 mμ - trough at 425 mμ) were plotted as a function of polylysine concentration, as shown in Figure 24, the curve showed a rapid rise in rotation at low concentration of the polypeptide, reached a maximum amplitude of 89,000° at polylysine concentration of 200 mμM (L/H = 7.8) then finally decreased to about 55,000° at higher polylysine concentration.

The ORD behavior of the CO-treated red complex likewise differed from those described earlier. The dispersion curve at 175 mμM polylysine (L/H = 6.8) showed a simple Cotton effect having extrema at 427 mμ (positive), 412 mμ (negative) and the inflection point at 421 mμ. Increasing the polypeptide concentration increased the rotations at both the extrema. However the increment at the positive extremum was much greater than at the negative extremum. Consequently, when the polylysine concentration reached 400 mμM or higher, the Cotton effect became

Figure 23. Spectropolarimetric titration of CO-heme a-poly-L-lysine as a function of poly-L-lysine concentrations. Buffer medium: 0.05 M phosphate containing 1% Emasol-1130, pH 11.6. The system contained 14.9 μM hematin a and various amounts of poly-L-lysine. The solution after dithionite reduction was bubbled with carbon monoxide for 1 minute before ORD measurements.

L/H: number of lysyl residues per heme molecule.

Figure 24. Titration curves of the systems, heme a-poly-L-lysine and CO-heme a-poly-L-lysine (in Emasol). Amplitudes are peak-trough values obtained from curves shown in Figures 22 and 23.

Reduced: heme a-poly-L-lysine.

Reduced-CO: carbon monoxide reacted heme a-poly-L-lysine.

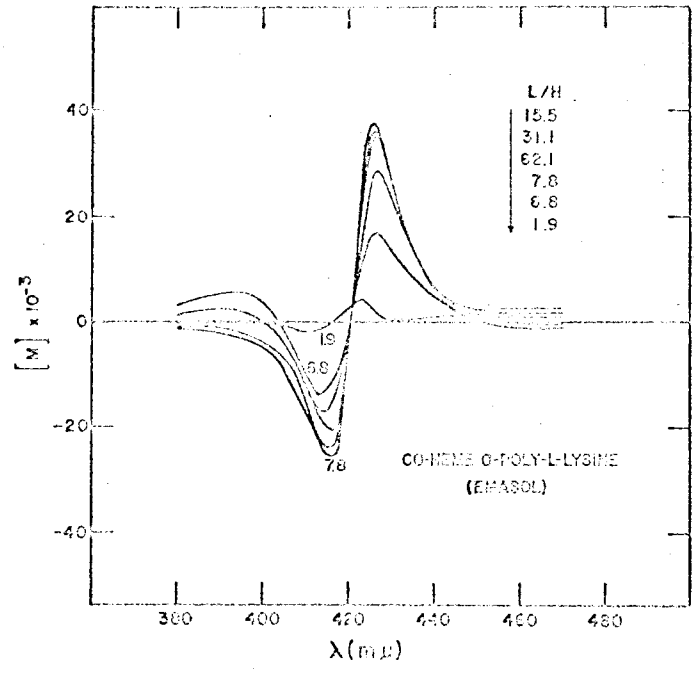
L/H
15.5
31.1
62.1
7.8
6.8
1.9
40
20
0
-20
-40
[M] x 10⁻³
1.9
6.8
7.8
CO-HEME G-POLY-L-LYSINE
(EMASOL)
300
400
420
440
460
480
λ(mμ)

Fig. 23

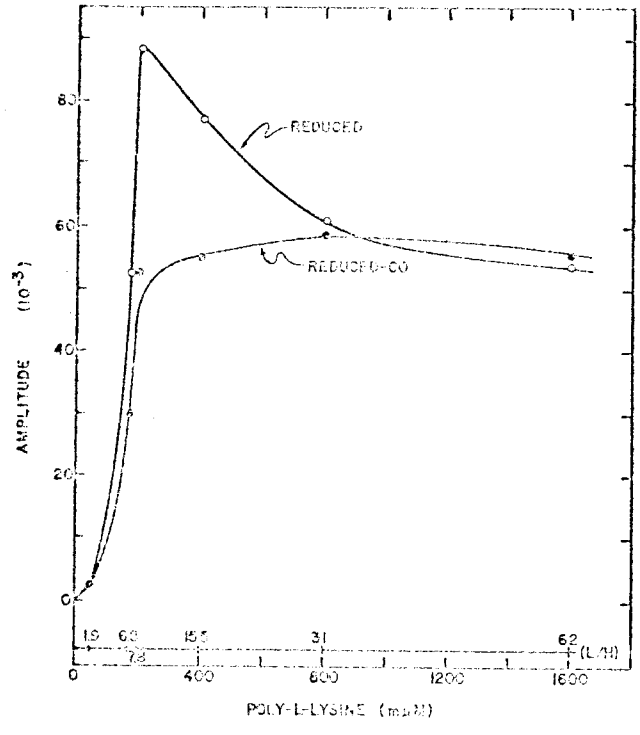
80
60
40
20
0
AMPLITUDE (10⁻³)
REDUCED
REDUCED-CO
1.9
6.8
7.8
15.5
31
62
(L/H)
0
400
800
1200
1600
POLY-L-LYSINE (mμM)

Fig. 24

unsymmetrical. Like the reduced form and those observed earlier, further increase in the polypeptide concentration decreased the magnitude of rotation at the positive extremum although much less in this case. The curve obtained by plotting the change in amplitude (peak at 427 mμ - trough at 412 mμ) against polylysine concentration is shown in Figure 24. In contrast to the reduced form, the plot showed a rapid increase in rotation at low polypeptide concentrations then levelled off at concentrations greater than 200 mμM (L/H = 7.8). The maximum amplitude was about 59,000° and decreased by less than 1% with further increase in polylysine concentrations. It is no doubt that heme a treated with Emasol-1130 as well as those without treatment, will interact with poly-L-lysine to form red complex but exhibiting quite different optical rotatory dispersion behaviors. Since Emasol-1130 acts as a dispersing agent, these differences perhaps reflect the different states of heme a, its heme to polymer as well as heme-heme interactions resulting from different modes of binding of the hemes to the polypeptide.

Spectropolarimetric Titrations of Protoheme-poly-L-lysine System -- The formation of protoheme-poly-L-lysine complex and its absorption spectra had been reported (4, 32). Stryer (61) showed that the hemin-poly-L-lysine complex formed at high concentration of poly-L-lysine, exhibited induced negative Cotton effect in the region of its Soret absorption maximum. The ORD properties of

the reduced complex (protoheme-poly-L-lysine) formed at low protoheme concentration and measured as a function of poly-L-lysine concentration, is shown in Figure 25. The anomalous dispersion curves exhibited a simple Cotton effect having a negative extremum at 435 mμ, a positive extremum at 418 mμ and an inflection point at 429 mμ. At the initial phase of the titration, the increase in rotation at both extrema was proportional to the amount of poly-L-lysine added. The increment gradually levelled off as the concentration approached 1200 mμM (L/H = 69.4) and remained at approximately the same magnitude of rotation even when 5-fold excess of poly-L-lysine was added. This saturation effect was likewise demonstrated when the amplitude of the Cotton effect was plotted against the poly-L-lysine concentration. This is illustrated in Figure 26. There was an almost linear increase in the amplitude rotation at low concentration of poly-L-lysine (500 mμM). It reached the maximum value (650,000°) at concentrations greater than 1000 mμM and remained at that level without being affected by further increase in poly-L-lysine concentration. From the titration curve and based on a model having 2 lysyl residues coordinated to the iron-atom of one protoheme molecule, the lysyl residues to heme ratio at saturation was found to be 11.5. This value is close to those reported in the literature (2).

Figure 25. Spectropolarimetric titration of protoheme-poly-L-lysine as a function of poly-L-lysine concentration. Buffer medium: 0.05 M phosphate, pH 11.6. Titration performed by mixing 10 μM protohematin with different amounts of poly-L-lysine. The solution was reduced with dithionite for 10 minutes prior to ORD measurements. L/H: number of lysyl residues per heme molecule.

Figure 26. Titration curve of the system, protoheme-poly-L-lysine.
Amplitudes are peak-trough values obtained from curves shown in Figure 25.

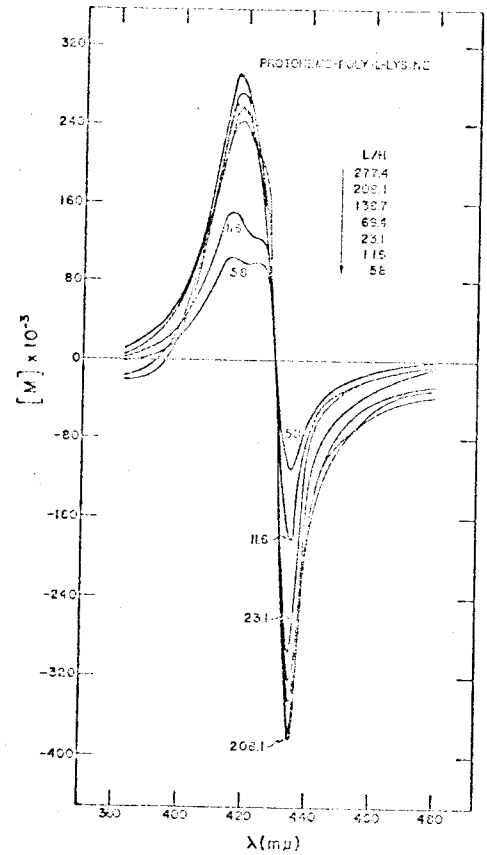
PROTONATED POLY-L-LYSINE
L/H
277.4
208.1
138.7
69.4
23.1
11.6
5.8
[M] x 10⁻³
320
240
160
80
0
-80
-160
-240
-320
-400
300
400
420
440
460
480
λ(mμ)

Fig. 25

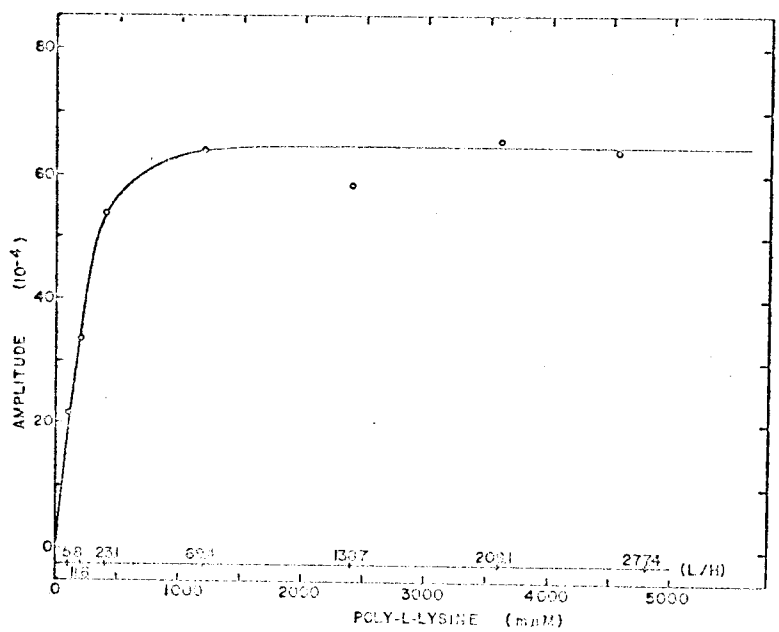
AMPLITUDE (10⁻⁴)
80
60
40
20
0
5.8
23.1
69.4
138.7
208.1
277.4
(L/H)
11.6
0
1000
2000
3000
4000
5000
POLY-L-LYSINE (mM)

Fig. 26

DISCUSSION

Optical rotatory dispersion measurements have been shown to be an effective and sensitive probe for changes in the conformation of a protein molecule in solution, particularly for alterations affecting an optically active chromophore. In the case of the alkali-denatured cytochrome oxidase, its ORD profiles in the Soret region appeared to have been changed so drastically when compared to those of the native enzyme (35, 65), that it provides a clear indication of conformational changes in the molecule as a result of alkali action. Judging from the change in sign and shape of the dispersion curves as compared to the Cotton effects of the native enzyme, the modifications probably resulted in juxtaposition of the heme molecules, perhaps close enough to bring about effective heme-heme interactions. The effect of alkali is drastic in view of the abrupt changes in the ORD and the rapid, irreversible loss of enzymic activities of the protein (40, 62). From the ORD behavior, it is not possible to evaluate whether the alkali acts specifically on only one of the cytochrome components of oxidase (i.e. cytochrome *a* or a_3). However, Mansley, *et al.* (44) had shown that cytochrome oxidase exposed to alkali, could bind twice as much CO as the native enzyme. This provides an indication that

at alkaline pH, cytochrome a is modified, perhaps to a form not dissimilar to cytochrome a_3. Indeed, the ORD profile of the denatured enzyme which showed no anomalous rotation in the oxidized state but a couplet-type dispersion curve for the reduced and CO-treated forms, can be interpreted as arising from one molecular species. It is no doubt that the changes in the extrinsic Cotton effects are related to the loss of enzymic activities as well as changes in the absorption spectra of the denatured enzyme.

It is perhaps not too surprising to observe that the ORD of alkali-denatured cytochrome oxidase differed from those exhibited by heme a-poly-L-lysine complex (cf. Figures 13 and 15). This is because the measurements which reflect the interactions involving heme-protein and heme-heme (if any), are very much affected by the extent of helix content in the protein as well as the states of aggregation and orientations of the heme molecules. Under conditions as those described in this study, poly-L-lysine has been shown to exist practically all in the α-helical configuration (1). Although no quantitative data on the helicity of alkali-denatured cytochrome oxidase are available, its helix content probably would be less than the value reported for the native enzyme (35, 59). This difference together with the different states of heme a probably contribute to the differences observed in the rotatory dispersion of

the denatured enzyme and the poly-L-lysine complexes.

On the other hand, there is a striking resemblance between the ORD of the alkali-denatured enzyme and heme a-poly-D-lysine complex. Both exhibited a deep trough flanked by two small peaks for their reduced as well as the CO-treated forms, although, however, the trough for the oxidase occurred at slightly longer wavelength (cf. Figures 13 and 16). Considering the fact that poly-D-lysine not only has a left-hand α-helical configuration but also a low molecular weight (4000-8000, ave. degree of polymerization, 60), the similarity of their dispersion curves is indeed surprising. Since the denatured enzyme exhibited rotations typical of all poly-L-amino acids in the UV-region, the possibility of having polypeptides or segments of polypeptide attached to the heme, to exist in the D-configuration, seems unlikely. Thus, it appears that the similarity of the dispersion curves resides in the mode of interaction of the heme a molecule with the polypeptide. The structure of heme a itself is inherently dissymmetric (78). The side chains are so arranged on the molecule that on one end of the porphyrin ring are located hydrophobic groups (vinyl and large alkyl) while on the other end, two negatively charged propionic acid side chains. The effective dipole moment arising from such a dissymmetric structure will therefore, generate optical rotation when coupled to the

helical structure of the polypeptide. However, the sign of the optical rotation will be dependent not only upon the sense of the helix but also the polarization of the interacting dipole moment. It is envisaged that in alkali-denatured oxidase, the direction of the dipole moment and hence the orientation of the heme molecule, is opposite to those heme a molecules bound in the poly-D-lysine complex. The result is that the induced rotation of the denatured enzyme bears the same sign as those of the poly-D-lysine complex. The answer to the question as to why a different orientation of heme a is favored in the denatured oxidase, resides perhaps, on the modes of binding of the heme molecule to the protein moiety. It has been demonstrated that heme a in alkali condition, undergoes ligand as well as Schiff-base reactions with lysine residues (62). It is therefore probable that the heme molecule has to orient itself in a direction opposite to that found in synthetic complexes, in order to have Schiff-base and ligand reactions with the lysine residues of the protein moiety.

Further indication of the different orientation of heme a molecule in the denatured oxidase comes from the observed rotatory behavior of the poly-L-lysine-heme a complex in the presence of Emasol-1130 (cf. Figure 22). The dispersion curve for the reduced form is exactly the opposite of the poly-D-lysine heme a

complex. This is due to different helical configuration of the polypeptide rather than the orientation of the heme molecule. In fact, the orientation of the heme molecule should be similar in both cases. An interesting difference between the observed rotation of the two synthetic complexes is the much greater magnitude of rotation for the poly-L-lysine heme a complex. This increase in rotation is perhaps related to the chain length of the polypeptide and the effective heme-polypeptide and heme-heme couplings. For poly-D-lysine, the average degree of polymerization is 60 which is about 10-fold smaller than the poly-L-lysine ($\bar{p}$ = 578). The dipole-dipole coupling between heme chromophores bound to the polypeptide is therefore very much more pronounced in the long polypeptide chain where more hemes are attached than the shorter poly-D-lysine. The different degree of coupling or perhaps the different mechanism of coupling is also reflected by the perturbation of CO on the ORD of these complexes. For the poly-D-lysine heme a complex, the dispersion curve appeared to be little affected by CO. However, the poly-L-lysine heme a complex underwent significant changes as evidenced from the conversion of the couplet-type dispersion curve to a simple positive Cotton effect which is unsymmetrical (cf. Figure 23).

The effect of Emasol-1130 on the ORD behavior of poly-L-lysine

heme a complex is clearly demonstrated by comparing the dispersion curves of the complex formed in the presence of Emasol-1130 (cf. Figure 22) with those formed without it (cf. Figure 19). Since Emasol-1130 has the ability to disperse heme aggregates that have been shown to exist in aqueous alkali medium (cf. Section 1), the different dispersion curves therefore reflect different modes of interactions arising from heme-heme as well as heme-polypeptide couplings in the two types of complexes. In the presence of Emasol where the heme molecules are monomeric dispersed, it is envisaged that the heme molecules are bound to the polypeptide, perhaps in a helical array, to form a superhelix. The points of attachment of the heme molecule to the polypeptide are the iron-ligand as well as the Schiff-base binding. The heme planes are probably oriented parallel to the screw sense of the polypeptide. The consequence of such a structure, as predicted by the Exciton-coupling model (63), will generate optical rotation having a prominent peak flanked by two small troughs and occurring in the region of its absorption band. An example is shown in Figure 22. In contrasts, heme a-poly-L-lysine formed without Emasol-1130, probably consists of a cluster of heme a molecules bonded closely together, differently oriented and probably localized irregularly along the polypeptide. The suggestion of such a structure is indicated from the ORD changes

following the spectropolarimetric titration of heme a with poly-L-lysine (cf. Figures 19 and 20). The changes suggest depolymerization of the heme a aggregates following the addition of more poly-L-lysine. The sequence of events that occur following the initial binding of a heme a aggregate to the polypeptide is the depolymerization of the aggregate by adjacent lysyl residues interacting through Schiff-base and ligand reactions. The resulting structure therefore consists of heme a molecules located close together on certain segment of the polypeptide and probably randomly oriented. Because of the asymmetric binding of the heme molecules and the strong dispersion interactions among the heme chromophores due to their close proximity, optical rotations giving symmetric Cotton effects are generated in the vicinity of its absorption band.

Based on the structural models of heme a-poly-L-lysine as discussed above, the certain peculiarities observed in the spectropolarimetric titrations of these systems can then be explained. As pointed out earlier, titration of heme a with poly-L-lysine with or without Emasol, does not give a saturation type of titration curve which is observed in spectrophotometric titrations of the same system (32). The reasons for the deviation is borne out in the structural model. For the complex formed in Emasol-1130, initial binding of monomeric dispersed heme a molecules to low concentra-

tion of poly-L-lysine, involves probably Schiff-base and one ligand coordination only. Because the hemes are linked in a helical array along the polypeptide (see earlier discussion of this section), maximum rotation is observed when optimum concentration of poly-L-lysine are added so that all the hemes are bound asymmetrically. Addition of excess poly-L-lysine then allows the 6th coordination site of the heme molecules to be linked to lysyl residues coming from other polypeptides. The resulting structure where heme a molecules are sandwiched between different polypeptide chains, reduces its asymmetry as a whole and therefore, decreases its magnitude of rotation. However, the basic pattern of heme a molecules linked in a helical array around certain poly-L-lysine chains still remains. This accounts for the retention of the shape of the dispersion curve but decrease in amplitude of rotation at high concentration of poly-L-lysine. Such a structure further explains the effect of CO on the ORD changes of this system. The results which are illustrated in Figure 23, showed that CO brings about a saturation-type of spectropolarimetric titration curve (cf. Figure 24). This is because CO, which is a good ligand for heme-iron, displaces the lysyl residues from the 6th coordination of the heme and hence destroys the symmetry of the sandwiched structure. The result is that addition of excess poly-L-lysine will

not affect the amplitude of rotation of the observed Cotton effects.

On the other hand, because the structure for heme a-poly-L-lysine formed in absence of Emasol-1130 is different from that just described, the effect of excess poly-L-lysine on these complexes is somewhat different. Here, the induced optical rotation is very much dependent upon the dispersion interactions occurring among the heme chromophores which are located close together. The effect of adding excess poly-L-lysine is to decrease such interactions, for example, through ligand reactions involving the 6th coordination site of the heme molecules, or Schiff-base or even displacing a heme molecule from one polypeptide chain to another because of unfavorable orientation of the heme plane or its side chains. Thus, the result is a decrease in the amplitude of rotation of the Cotton effects following addition of excess poly-L-lysine. This "dilution" effect of poly-L-lysine holds true in both the reduced form as well as the CO-treated heme a-poly-L-lysine complex. The results are shown in Figures 19, 20 and 21.

Finally, the spectropolarimetric titration of protoheme-poly-L-lysine complex as a function of polylysine concentrations also shows a saturation type of titration curve (cf. Figure 26). The fact that protohemes have been shown to exist as small aggregates in aqueous solutions (3, 60) and that its complex (protoheme-poly-L-

lysine) exhibits a simple negative Cotton effect with very large amplitude (cf. this section on results), would suggest that the modes of binding of protoheme to the poly-L-lysine is somewhat similar to that described for heme a-poly-L-lysine complex formed in the absence of Emasol-1130. However, in contrast to heme a, protoheme contains no formyl group as side chain and therefore does not undergo Schiff-base reaction. It follows then that ligand reactions can occur on both sides of the heme plane with equal probability. In fact, it seems highly probable that all the protoheme molecules are sandwiched between two poly-L-lysine chains as soon as they interact with the polypeptides. Such a structure has been considered feasible by Blauer (2). Under these conditions, it is obvious that addition of excess poly-L-lysine will not affect the amplitude of rotation of the Cotton effect. This is demonstrated by the results depicted in Figures 25 and 26.

3. ATTEMPTED RECONSTITUTION OF CYTOCHROME c OXIDASE FROM AN APO-CYTOCHROME c-OXIDASE PREPARATION

INTRODUCTION

Although MacMunn (43) discovered cytochromes in 1886 (then known as histohematins or myohematins), their importance was not recognized until Keilin in 1925 (24) reported their wide distribution in the biological kingdom and their physiological function as a hydrogen (electron) carrier in cellular respiration. The definitive studies of Keilin not only rationalized and united the two seemingly incompatible theories (70, 73) concerning the oxidation of substrates and the activation of oxygen in cellular respiration but also established the concept of the electron transport chain where the cytochromes are visualized to mediate between the substrates and oxygen. The idea of the respiratory chain is now overwhelmingly demonstrated (11, 29). The general arrangement of the respiratory chain is composed of dehydrogenases such as succinic dehydrogenase and NADH-dehydrogenase, both of which accept electrons from the substrates, cytochromes b, c_1, and c, then finally the terminal enzyme, cytochrome oxidase which interacts with oxygen. The complexity of cytochrome oxidase was first recognized by Keilin and Hartree (25) who concluded from their

extensive studies of the effect of various inhibitors on the spectra of cytochrome oxidase, that the enzyme was in fact composed of two intimately related entities which they named cytochromes a and a_3. Early attempts to isolate cytochrome oxidase were made by Yakushiji and Okunuki (75) who showed that the enzyme could be solubilized from muscle tissues in the presence of both cholate and ammonium sulfate. Utilizing the same principle, Yonetani (76) was able to prepare cytochrome oxidase of reasonable high purity to enable him to study the spectral and kinetic properties of the enzyme and from which he deduced the spectral properties of the two entities, namely cytochrome a and a_3. In the intervening years that follow, extensive studies on purified cytochrome oxidase prepared from various sources had been reported (12, 29, 30, 41, 51, 76). Among these studies, the investigations of the CO-binding capacity of the enzyme (18, 44, 68) and their redox behaviors in the presence of chelating agents and inhibitors (67) greatly enhanced our knowledge of the enzyme. It is now generally accepted that the functional unit of cytochrome oxidase is composed of an equal amount of cytochromes a and a_3 as well as copper. Despite the fact that the separate entity of cytochromes a and a_3 has been overwhelmingly demonstrated by spectral studies (51, 69), yet all attempts to isolate them into two separate entities have not been

successful. A technic that has proved invaluable to the investigations of enzyme systems is the restoration of enzymic activities through reconstituting the enzyme system by putting together their respective components. Thus Nair and Mason (53, 54) successfully restored the enzymic activities of a partially copper-depleted cytochrome oxidase preparation by incubation of stoichiometric amount of cuprous acetonitrile perchlorate. Based on the same approach, attempts were made to study the possibilities of reconstituting the enzymic activities of a copper-depleted and heme-partially removed cytochrome oxidase preparation. The term "Apo-cytochrome c-oxidase" or "Apo-oxidase" refers to a preparation containing about 50% of the original heme groups and insignificant amount of copper.

EXPERIMENTAL PROCEDURES

Materials

Purified cytochrome oxidase was prepared according to the method developed by King, Kuboyama and Takemori (30). The purity of the enzyme was gauged by the index $A_{605\ m\mu}/A_{554\ m\mu}$ of the reduced enzyme. The ratio was usually greater than 2 indicating the absence of other cytochromes such as $\underline{c}_1$, $\underline{b}$ and $\underline{c}$. Concentration of the enzyme was determined by using an extinction coefficient of 12 ($mM^{-1}\ cm^{-1}$) for the reduced minus oxidized absorption at 605 mμ.

Hematin $\underline{a}$ was extracted from purified cytochrome oxidase by acid acetone. Details of the extraction procedure was described by Takemori and King (62). The ethereal extract of the heme solution was finally evaporated to dryness and stored in vacuum, away from light until used. Determination of the hematin $\underline{a}$ concentration was made from its pyridine hemochromogen formed in 20% pyridine, 0.05 N NaOH solution. An extinction coefficient of 27.4 ($mM^{-1}\ cm^{-1}$) at 587 mμ was used for computation (16).

Methods

Cuprous acetonitrile perchlorate ($Cu^{1}(CH_3CN)_4 \cdot ClO_4$) was prepared by the method of Hemmerich and Sigwart (21). A limited

amount of cuprous oxide was mixed with excess amounts of perchlorate and acetonitrile. The suspension was heated in water bath until it turned into colorless solution. Chilling the solution at this stage yielded crops of tiny, white needle-like crystals of the cuprous compound. The crystals were filtered through sintered glass, washed twice with cold acetonitrile-perchlorate solution and then dried under vacuum. The product was stored in nitrogen.

For the preparation of apo-cytochrome oxidase, the native enzyme after having been initially precipitated in 40% ammonium sulfate, was dissolved in 2% cholic acid previously neutralized with concentrated ammonium hydroxide. The dark greenish solution was dialyzed against water for three hours at 4° C. In a typical preparation, 2 ml of the above enzyme solution of approximately 1.5% protein concentration, were added dropwise into 100 ml of acetone containing 0.108 N HCl and precooled to about -15° C in a dry ice-acetone bath. The solution was stirred rapidly for about 5 to 15 minutes before being centrifuged. The precipitate was immediately dissolved in ice-cold water. The aqueous solution was then dialyzed against water for 3 hours before being used for later experiments.

To reconstitute the holo-enzyme, the apo-oxidase solution

(3-5 mg protein/ml) was incubated with equal molar concentration of hematin a for 60 minutes at 4° C and pH 7.0. At the end of incubation, the solution was precipitated with 50% ammonium sulfate and dissolved in 0.05 M phosphate buffer containing either 1% cholate or 1% Emasol, pH 7.4. Stoichiometric amount of cuprous acetonitrile perchlorate were added and the solution incubated for 2 hours at 4° C. After incubation, the solution was precipitated and the precipitate again suspended in 0.05 M phosphate containing either 1% Emasol-1130 or 1% cholate.

A variation of the reconstitution experiments involved using excess amounts of hematin a and cuprous acetonitrile perchlorate. Appropriate control experiments were included.

Determinations of iron and copper were performed according to the procedures described by King, et al. (28). Protein concentration was determined by biuret. Enzyme activities were measured in a G.E. oxygraph fitted with a Clark electrode. The 2 ml assay system contained 1% Emasol-1130 in 0.05 M phosphate, 2 mg/ml asolectin, 2×10^{-5} M cytochrome c, 0.03 M sodium ascorbate, 2×10^{-4} M sodium ethylenediamine-tetra-acetic acid and various concentrations of enzyme.

RESULTS

Iron and Copper Content of Apo-cytochrome c-Oxidase -- The results of chemical analysis of iron and copper content in the acid acetone extracts and the residues (Apo-oxidase) are shown in Table V.

TABLE V

Chemical Analysis of Acid Acetone Extracts and Residues of Cytochrome c Oxidase

	Distribution of Iron		Distribution of Copper	
	μatom	%*	μatom	%*
Sample I				
Acid acetone extract	0.32	63	0.45	90
Residues (Apo-oxidase)	0.22	44	0.04	7
Sample II				
Acid acetone extract	0.32	63	0.45	89
Residues (Apo-oxidase)	0.19	38	0.02	4

*In terms of amount of material used.

It is evident that practically all the copper and about 50% of the iron were extracted from the enzyme by acid acetone treatment.

The apo-oxidase therefore, contained only approximately 50% iron and very little amount of copper.

Interactions of Apo-Cytochrome c-Oxidase with Hematin a -- Apo-cytochrome c-oxidase solution obtained after dialysis was usually clear and acidic. The pH of the solution was 3.7-4.0. Addition of buffer or adjustment of pH to neutrality always causes turbidity. Figure 27 shows the difference spectra of apo-oxidase in the presence of hematin a in 0.05 M phosphate buffer containing 1% cholate. The spectra appeared to behave differently from those exhibited by free hematin a in the same buffer medium as shown in Figure 28. Thus, when reduced by dithionite, apo-oxidase with heme a showed a hemochrome-type spectra with absorption peaks at 599 mμ and 442 mμ. Free heme a gave but only a Soret peak at 427 mμ. Saturating the solution with CO gave, for the apo-oxidase-heme a system, absorption maxima at 603 mμ and 436 mμ while free heme a showed absorption maxima at 602 mμ and 411 mμ. Although the spectral behavior of apo-oxidase-heme a system indicated interactions between apo-oxidase and heme a, the spectral profile was not identical to those exhibited by intact cytochrome oxidase, as shown in Figure 29. The most distinct difference between the two systems is the effect of CO. For heme a-apo-oxidase, the a-maximum was shifted from 599 mμ to 603 mμ (red shift)

Figure 27. Difference spectra of apo-oxidase in the presence of hematin a.

The system contained 0.32 mg/ml apo-oxidase and 10 μM hematin a in the sample cuvette and the same concentration of apo-oxidase in the reference cuvette. Buffer: 0.05 M phosphate containing 1% cholate, pH 7.4.

Oxidized: system as such.

Reduced: reduction by dithionite.

CO-reduced: solution after reduction was bubbled with CO for 1 minute.

Figure 28. Absorption spectra of hematin a in 0.05 M phosphate containing 1% cholate, pH 7.4.

Hematin a concentration: 10μM.

Oxidized: system as such.

Reduced: reduction by dithionite

Reduced-CO: Solution after reduction was bubbled with CO for 1 minute.

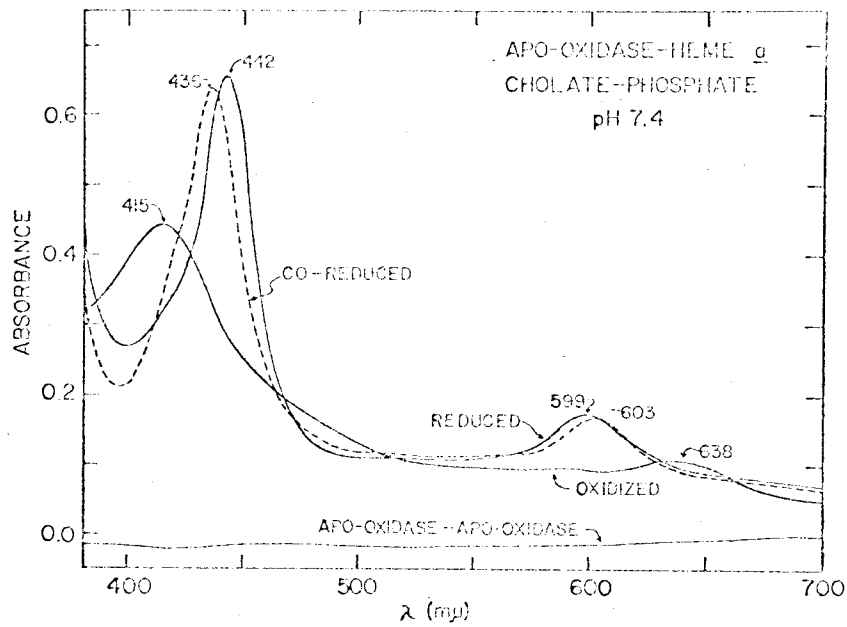
APO-OXIDASE-HEME a
CHOLATE-PHOSPHATE
pH 7.4
442
435
415
CO-REDUCED
599
603
638
REDUCED
OXIDIZED
APO-OXIDASE - APO-OXIDASE
ABSORBANCE
0.0
0.2
0.4
0.6
400
500
600
700
λ (mμ)

Fig. 27

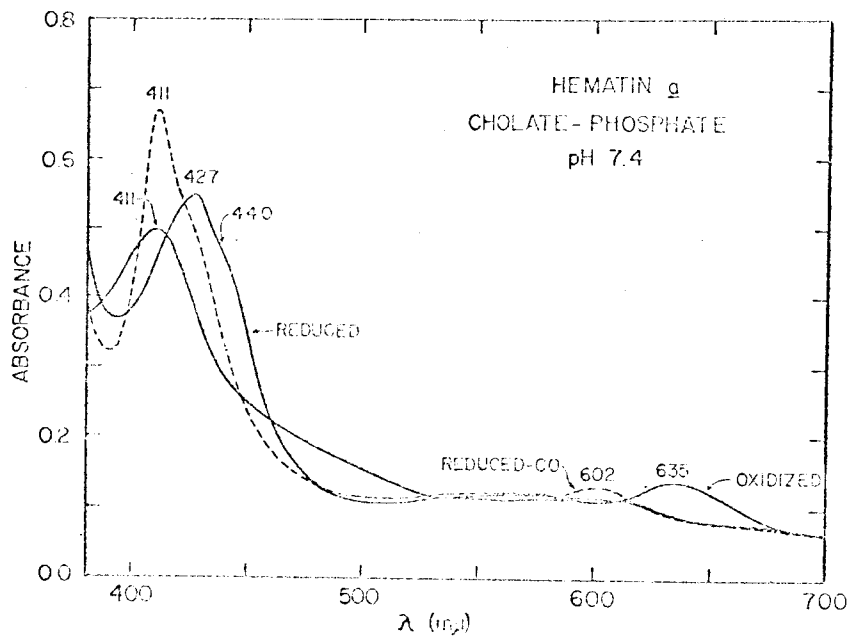
HEMATIN a
CHOLATE-PHOSPHATE
pH 7.4
411
427
411
440
REDUCED
REDUCED-CO
602
635
OXIDIZED
ABSORBANCE
0.0
0.2
0.4
0.6
0.8
400
500
600
700
λ (mμ)

Fig. 28

Figure 29. Absorption spectra of intact cytochrome c oxidase in 0.05 M phosphate containing 1% cholate, pH 7.4.

Cytochrome oxidase concentration: 7.2 μM.

Oxidized: system as such.

Reduced: reduction by dithionite.

Reduced-CO: The solution after reduction was bubbled with CO for 1 minute.

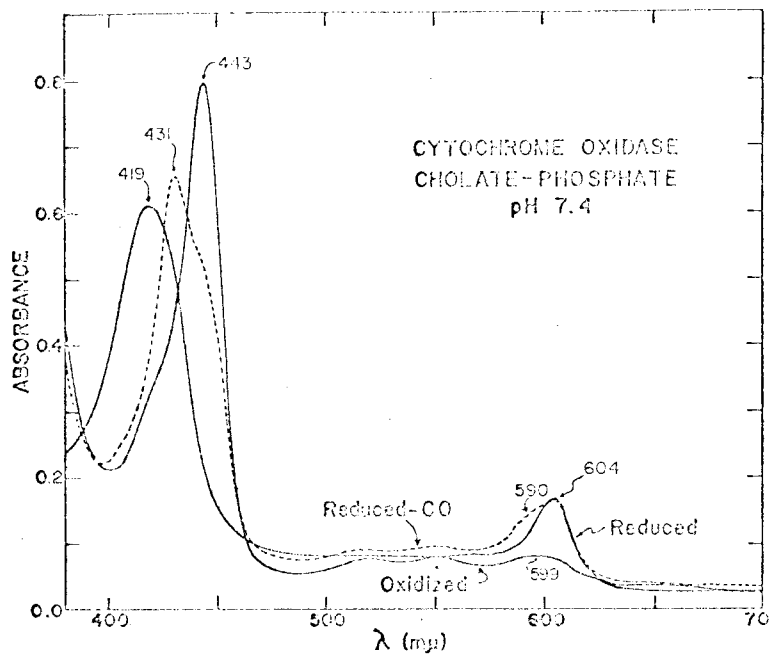
CYTOCHROME OXIDASE
CHOLATE-PHOSPHATE
pH 7.4
443
431
419
604
590
599
Reduced-CO
Reduced
Oxidized
ABSORBANCE
0.0
0.2
0.4
0.6
0.8
400
500
600
700
λ (mμ)

Fig. 29

while, in intact cytochrome c oxidase, a blue shift (604 mμ to 590 mμ) was evident.

Attempts were made to reconstitute the holo-enzyme by incubating the apo-oxidase preparation with either stoichiometric amounts of hematin a and cuprous acetonitrile perchlorate or excess amount of one of them. The resulting complex did not show any enzymic activities. Likewise, when reconstitution was made according to the method of Kuboyama and King (36), where the apo-enzyme was incubated in the presence of ascorbate-cytochrome c, no enzymic activities were restored.

DISCUSSION

The ability of acid acetone to remove only one-half of the heme from cytochrome c-oxidase is indicative of the two different modes of binding of the heme groups in the enzyme. This is in accord with observations reported in the literature (52). The hemes extracted by this treatment, are presumably from those of cytochrome a_3. The results of the chemical analysis together with the known fact that acetone also removed a certain amount of lipids from the material, suggest that acid acetone is too drastic for the enzyme. The apo-protein prepared under this condition is probably denatured. This accounts for the lack of enzymic activities of the reconstituted holo-enzyme. For future experimentations, reagents that are mild to the protein moiety of the enzyme but are specific in removing either the heme groups or the copper components will be recommended.

GENERAL DISCUSSION

The results from the optical rotatory dispersion studies of hematin a fully established the asymmetric structure of hematin a molecule and its existence as aggregates in aqueous medium. The rotational strength of the molecule appears to be derived from the heme-iron which is the center of dissymmetry and is supplemented by the formation of aggregates and by the orderly structure of the molecule as a result of interactions between the long alkyl group at side chain-2 of the heme plane with other side chains or solvent molecules. However, no optical rotation can be attributed to the asymmetric carbon atoms that are deduced from structural studies (9, 19, 20, 42). Their resolutions perhaps require much more sensitive instrumentations.

The complex formed between heme a and poly-L-lysine has served as model for the studies of alkali denatured cytochrome c oxidase. The ORD results of the various polylysine-heme complexes and the alkali denatured enzyme provide further information concerning the mode of interaction between the heme groups and the ligands on the z-coordinate of the heme-iron. For the denatured enzyme, the experimental results suggest (1) drastic conformational changes that bring about loss of enzymic activities, (2) close alignment of the heme groups to give effective heme-heme interactions

and (3) the polarization of the heme plane is opposite to those found in the poly-L-lysine heme a complexes.

The separation of cytochromes a and a_3 has been the ultimate objective of many researchers in this field. It is hoped that the results presented in this dissertation will help provide a better foundation whereby sophisticated experiments will be developed for their separation in the future.

SUMMARY

1. Optical activity of hematin a was demonstrated in aqueous solution.

2. The complexities of the ORD of hematin a were shown by the effects of various parameters (concentrations, pH, sodium chloride and organic solvents) on the dispersion behaviors of the molecule. From these results were deduced the various factors (aggregation, ligand reaction, stereochemistry and conformation of the molecule) contributing to the observed optical rotations.

3. Aggregation of hematin a in aqueous solution was confirmed by sedimentation studies.

4. The ORD of alkali denatured cytochrome c oxidase was compared with those of heme a-poly-L-lysine and heme a-poly-D-lysine complexes under various conditions.

5. From spectropolarimetric titrations of the formation of heme a-poly-L-lysine in the presence and absence of Emasol-1130 and of protoheme-poly-L-lysine, the relative contributions of heme-heme and heme-protein interactions to the observed optical rotations were discussed. Based on these results, the mode of binding and the orientation of the heme groups in the denatured enzyme were hypothesized.

6. Attempts to reconstitute the holo-enzyme from an apo-cytochrome c oxidase preparation and hematin a were described.

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