Studies on Cytochrome Oxidase

III. LIGAND REACTIONS AND SCHIFF BASE FORMATION OF HEME a*

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SUMMARY

Hematin a isolated from purified cytochrome oxidase reacts with poly-L-lysine and with proteins of high lysine content, such as histone, albumin, and globin, in neutral and alkaline media. At pH 11.6, spectral characteristics of these synthetic complexes are strikingly similar to those of cytochrome oxidase. Although absorbance indices differ, the positions of the band maxima are virtually the same in the oxidized, the reduced, the carbon monoxide-reduced, the borohydride-treated, and the borohydride-treated followed by carbon monoxide reduction forms. After borohydride reaction, the heme a in these complexes as well as in cytochrome oxidase is no longer extractable by acid-acetone.

On the other hand, these characteristics are not shared when hematin a is replaced by protohematin. Hematin a does not react with histidine, lysine, glutamic acid, aspartic acid, proline, asparagine, polyglutamic acid, polyproline, or protamine in either neutral or alkaline media, or with polylysine in alkaline solutions under conditions similar to those used for polylysine, i.e. at less than 2 mg of nitrogenous compound per ml. However, the spectra of heme a in the presence of 5% lysine (but not other free amino acids tested, including methionine, histidine, and tyrosine) closely resemble those of the heme a-polylysine complex. Although hematin a reacts with polysarcosine and polyhistidine, the reaction differs from that with polylysine.

From the data presented here, together with the behavior of cytochrome oxidase described previously (30), it is tentatively concluded that at least 1 lysine residue of cytochrome oxidase coordinates with the heme iron at pH 11.6, and another lysine residue forms a Schiff base with the formyl group on the porphyrin nucleus. The justification for the conclusion, along with certain reservations, has been pointed out.

A voluminous and informative literature is available on the interaction between protohematin and ligands (e.g. References 2, 13, and references cited therein’). Probably because of the instability of hematin a, papers on its reactions with ligands are less numerous (e.g. References 13-23). To our knowledge, only two schools have investigated the behavior of hematin a toward globin and albumin (16-19). Furthermore, these studies are mainly confined to spectral and, to a lesser degree, magnetic properties. However, no investigation of the interactions between hematin a and synthetic polypeptides has been reported prior to this communication.

Kiese and Kura (16) and Morell, Barretto, Clezy, and Lemberg (17) have found that after alkali treatment of the artificially formed heme a-protein complexes, the a-bands of their absorption spectra between 590 and 596 mu shift to between 573 and 575 mu. Lemberg et al. (14, 17, 24) have explained that these spectral changes are due to the formation of a Schiff base between the formyl side chain of the heme a and an amino group of the protein. The Schiff base formation has been used to explain the spectral characteristics of cytochrome oxidase in alkaline media (25-30). This explanation has been greatly strengthened by the experimental demonstration of the fixation of heme a to the protein moiety of cytochrome oxidase after borohydride hydrogenation (29, 30). Interestingly enough Matsubara, Orii, and Okunuki have found that cytochrome oxidase is rich in lysine (31).

With these considerations in mind and in an attempt to shed some light on the identity of the particular amino acid (of the protein moiety) which coordinates with the heme, we have investigated the reaction between hematin a (and also protohematin) and polylysine (and also related compounds), examining their spectral characteristics, their behavior in borohydride hydrogenation, and their enzymic properties. This paper reports our results. Preliminary notes on some facets have appeared (32, 33).

EXPERIMENTAL PROCEDURE

Materials—Hematin a was prepared from purified cytochrome oxidase as detailed previously (30). The hematin a solution used in this paper was freshly prepared by dissolving dried samples in phosphate buffer containing 1% Emasol 1130. Emasol was obtained from Kao Soap Company, Tokyo. The native globin was

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isolated from freshly prepared crystalline ox oxyhemoglobin. The method used was similar to that originally developed by Rossi Fanelli, Antonini, and Caputo for Palamyssarda (34).

Fresh ox blood which was first diluted with 2 volumes of 1% sodium chloride was washed repeatedly with 1% sodium chloride in centrifuge. It was then hemolyzed with an equal volume of distilled water. The mixture was filtered with the aid of Celite and the filtrate was dialyzed against 70% ammonium sulfate for 24 hours at 4°. The resulting dialysate was kept in a Petri dish for crystallization at 4°. The hemoglobin crystals were dissolved in water to give a concentration of approximately 6%. The solution was dialyzed against water for 3 days with 10 changes of water. Six milliliters of 1% hemoglobin solution were added to the solution. The precipitate was harvested by centrifugation at -20°F which had been cooled to -20°. The solution was stirred rapidly. The precipitate was harvested by centrifugation at -20°F and then dissolved immediately in cold water. The solution was dialyzed against water for 6 hours, then 0.0016 N NaHCO₃ for 20 hours, and finally 0.02 N phosphate buffer, pH 7.0, for 24 hours. The dialysate was centrifuged. The clear, colorless supernatant liquid was used as the source of native globin. The clear solution was stable and could be stored at 2-5° for a week without change of properties. The globin content was determined spectrophotometrically using A₅₅₀ = 8.5. The "nativness" of the isolated globin was ascertained by the reconstitution technique with protohematin on the basis of spectral behavior and spectrophotometric titration (34, 35).

Four samples of poly-lysine in the form of hydrochloride with degrees of polymerization of 450, 578, 586, and 1,640 and molecular weights of 70,000, 74,000, 75,000, and 210,000, respectively, other poly-L-amino acids, and crystalline protohematin were procured from the Biophysics Department of The Weizmann Institute of Science. Other chemicals were obtained from commercial sources.

Methods—Concentrations of hematin a and protohematin were determined (30) by the pyridine-hemochromogen method in a system which contained 20% pyridine, 0.05 N NaOH, and a few grains of sodium dithionite; the reduction time was between 5 and 10 min. Activities of cytochrome oxidase and tetra-chlorohydroquinone oxidase were measured polarographically at room temperature (1). Direct chemical analysis for heme iron was performed as described earlier (30).

Spectrophotometric measurement was conducted at room temperature (22-25°) in a cuvette of 1-cm optical path in a Cary model 11 recording spectrophotometer or a Zeiss PMQ III manual spectrophotometer. Determination of pH was made by a Beckman Zeromatic pH meter; when more precise values were needed, a Beckman "expansion scale" pH meter was used. Other methods and manipulations are detailed in legends of appropriate tables and figures.

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

RESULTS

Interaction of Hematin a and Protot hematin with Polylysine

Spectral Characteristics of Heme a and Prot heme—Hematin a isolated from purified cytochrome oxidase according to the method described previously (30) was not easily soluble in water but could be slowly rendered into solution by Emasol-phosphate buffer at neutrality. The process of solution was greatly facilitated by first dissolving the hematin in a minimum volume of 0.01 N sodium hydroxide and then dialyzing with any suitable aqueous solvent without Emasol.

Since successive treatments of purified cytochrome oxidase with acetone and acetone-ether were used prior to the acid-acetone extraction of hematin a (30), the product was evidently free of lipid. It was not contaminated by other iron-porphyrin compounds because the starting material contained only hematin a free of mesohematin, protohematin, and "mitochrome" as judged by the A₆₆₅/A₄₅₄ ratio of the dithionite-reduced oxidase and by other criteria. The specific pyridine-hemochromogen method was employed for the determination of hematin a, and in turn its molar concentration was used as the basis for computation. As shown in Curve IV, Fig. 1B, the pyridine-hemochromogen spectrum likewise did not indicate the slightest abnormality or contamination with other hemes. In view of these facts, it was felt that the quantitative values reported in this paper were reliable.

Absolute spectra of hematin a in neutral media and at pH 11.6 are given in Fig. 1, A and B. Reduced heme a was not stable, as witnessed by the decrease of the absorbance on standing. A decrease of as much as 10% in the Soret region was observed in 30 min.

In phosphate buffer, pH 7.0, hematin a showed a Soret maximum at 406 m, with a relatively wide band, and a broad α-band centered at 633 m. The reduced form exhibited a pronounced hyperchromicity in the Soret region, with a peak at 430 m; the α-maximum centered around 633 m became even less distinct than in the oxidized form. Prolonged contact with dithionite further broadened the α-band to between 575 and 610 m, and on occasion the Soret peak was observed at 410 m. Hematin a at pH 11.6 possessed a broad α-peak at 633 m and a γ-peak at 405 m. Dithionite reduction further widened the α-band to between 575 and 610 m, but sharpened the Soret band to 436 m. These maxima of heme a were somewhat affected by the time of the reaction with dithionite (cf. Table I). Spectra of the pyridine-hemochromogen of heme a showed distinct maxima at 587 and 431 m (Curve IV, Fig. 1B).

In the literature, values of absorption maxima of hematin a are somewhat varied. This fact is not surprising because, usually, workers have used media containing different nitrogenous compounds of unspecified concentrations and the reaction time with dithionite is not described. It is known that hematin a forms hemochromogens but that the extent of the formation is dependent upon both the nature and concentrations of the ligands. Moreover, a large excess of dithionite "modifies" the heme. In view of these complications, we used phosphate buffer and did not allow the reduction by dithionite to go beyond 10 min unless otherwise specified. A large excess of dithionite was always avoided. Since our aim was not the study of hematin a per se, these variables were not further examined in detail.

Absorption maxima of hematin a and protohematin with an absorbance index in neutral and alkaline media are summarized in Table I. The absorbance was computed on the basis of 27.4 mm⁻¹ cm⁻¹ (30) at 587 m and 34.4 mm⁻¹ cm⁻¹ (Reference 11, page 240) at 556 m for the pyridine-hemochromogens of heme a and protoheme, respectively.
Fig. 1. Absolute absorption spectra of hematin a and the pyridine-hemochromogen: A, 14.5 μM hematin a in 20 mM phosphate buffer, pH 7.0; B, 14.5 μM hematin a in 0.1 M phosphate buffer, pH 11.6. Curve I, oxidized; II, dithionite-reduced (5- to 10-min reduction); III, base-line; IV, absolute absorption spectrum of the pyridine-hemochromogen of heme a. The system for Curve IV contained 5.8 μM hematin a in 20% pyridine and 0.05 N NaOH, with a few grains of sodium dithionite; reduction time, 10 min.

TABLE I
Main absorption maxima of hematin a and protohematin

The solvent used was 20 mM phosphate buffer, pH 7.0, or 0.1 M phosphate buffer, pH 11.6. Whenever carbon monoxide was used, the solution was bubbled with the gas for 1 to 2 min. The borohydride reduction was conducted at 0-4°C for 3 hours.

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH 7.0</th>
<th>pH 11.6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-Maximum</td>
<td>γ-Maximum</td>
</tr>
<tr>
<td></td>
<td>λ (μm) e μm⁻¹ cm⁻¹</td>
<td>λ (μm) e μm⁻¹ cm⁻¹</td>
</tr>
<tr>
<td>1. Hematin a, as such</td>
<td>653 7.9</td>
<td>406 46.6</td>
</tr>
<tr>
<td>2. System 1 + dithionite</td>
<td>~406 8.6</td>
<td>430 66.1</td>
</tr>
<tr>
<td>Reaction time, 5-10 min</td>
<td>430 66.1</td>
<td>460 6.5</td>
</tr>
<tr>
<td>Reaction time, 60 min</td>
<td>575-610 6.6</td>
<td>496 6.6</td>
</tr>
<tr>
<td>3. System 2 + CO</td>
<td>567 9.4</td>
<td>424 56.7</td>
</tr>
<tr>
<td>4. System 2 + borohydride</td>
<td>~410 8.9</td>
<td>~410 6.4</td>
</tr>
<tr>
<td>5. System 4 + CO</td>
<td>563 5.0</td>
<td>407 35.7</td>
</tr>
<tr>
<td>6. Protohematin, as such</td>
<td>~410 6.4</td>
<td>~410 6.4</td>
</tr>
<tr>
<td>7. System 6 + dithionite</td>
<td>520-600 4.6</td>
<td>407 111.1</td>
</tr>
<tr>
<td>8. System 7 + CO</td>
<td>564 10.4</td>
<td>407 111.1</td>
</tr>
<tr>
<td>9. System 7 + borohydride</td>
<td>Not made</td>
<td>~410 6.4</td>
</tr>
<tr>
<td>10. System 9 + CO</td>
<td>Not made</td>
<td>~410 6.4</td>
</tr>
</tbody>
</table>

*Dashes indicate that the peak was very broad.

The oxidized form showed a very broad band in the visible region and a broad maximum at about 400 μm. Upon reduction with dithionite, the broad γ-peak shifted to 424 μm (absolute spectra not shown). The difference spectra at pH 6.4 and 7.8 are shown in Fig. 2.

Interaction of Hematin a and Poly-Lysine in Alkaline Media—
When hematin a was mixed with polylysine in alkaline buffer, a greenish solution was formed. The absorption spectra are given in Fig. 3. Upon reduction by dithionite, the solution became wine-red. Distinct absorption maxima of the reduced complex were seen at 574 and 428 μm. These spectral properties of the heme α-polylysine complex are practically the same as those of cytochrome oxidase reported previously (30). When cytochrome oxidase is incubated in alkaline media, a stable spectrum is formed with maxima at 428 and 574 μm in the dithionite-reduced state, and at 407 μm in the oxidized form (30). These spectral
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Changes have been interpreted as the result of the formation of a Schiff base between the formyl group of heme α and an amino group of the protein moiety of cytochrome oxidase.

Effect of pH and Time on Spectral Properties of Heme α-Polylysine Complex—Since the characteristics of the absorption spectra of the heme α-polylysine complex are dependent upon the pH of the medium, the pH-dependent reversible change of spectra was examined. At neutral pH, the heme α-polylysine complex showed absolute spectra with broad bands. Upon addition of alkali to pH 11.6, the spectra changed to those shown in Fig. 3. Further neutralization of the alkalinized solution reversed the spectral characteristics to those observed at neutrality. This reversibility resembles that of the change in the second phase (30) of the reaction for cytochrome oxidase.

As described above, the spectral characteristics of the complex differed in neutral media from those at pH 11.6. Spectra at several pH values were thus investigated. As shown in Fig. 2 for the difference spectra (the dithionite-reduced form minus the oxidized) of the complex, the absorbance at 574 nm and at 429 to 431 nm increased markedly with the increase of the pH of the system. A distinct isosbestic point at 446 nm was observed.

A reaction time of a few minutes between hematin α and polylysine at pH 11.6 was sufficient to attain more than 90% of the maximal absorbance at 574 nm. The maximal value developed within 10 min. In the case of cytochrome oxidase, some time must be allowed for the reaction to attain the maximal formation of the 428 and 574 nm peaks by the action of alkali (30). These peaks are attributed to the Schiff base formed between the formyl side chain of the heme and an ε-amino group of the protein moiety. With respect to time as well as pH required, cytochrome oxidase differs slightly on the quantitative scale from the synthetic complex. In cytochrome oxidase, the extreme change in protein conformation from its neutral state is apparently necessary to liberate some free amino groups for the formation of the Schiff base. On the other hand, since polylysine has more nonprotonated ε-amino groups than the oxidase, the chances for the reaction may be greater, so that a shorter time is observed in the Schiff base formation.

It is well known that the helicity of polylysine is greatly dependent upon the pH of the solution (37). As depicted in Fig. 4, the pH profile for the absorbance of the Soret maximum of the heme α-polylysine complex approximated that for the helicity of polylysine. The disparity of less than 0.5 pH unit may be apparent rather than genuine because the experimental points on the original curve (37) of the helicity are somewhat scattered and the precision of the determination of the optical rotatory dispersion is not high. This observation strongly indicates that the spectral behavior of the complex depends not only on the nature of the ligand but also on its conformation.

Effects of Carbon Monoxide and Cyanide on Heme α-Polylysine Complex—The effect of carbon monoxide on the absorption spectrum of the heme α-polylysine complex in alkaline medium is shown in Fig. 3. It can be seen that the α-band was shifted from 574 to 584 nm, and the Soret peak, from 428 to 424 nm; the β-band became discernible at 544 nm. These changes are again reminiscent of those of cytochrome oxidase reported previously (30). Carbon monoxide also affected the spectrum of the complex in neutral medium; a blue shift to 414 nm and hyperchromicity in the Soret region were observed.

The effect of cyanide on the absorption spectrum of the com-

Fig. 2. Effect of pH on the difference spectrum (reduced minus oxidized) of the heme α-polylysine complex. The system contained 9.2 μM hematin α and 0.5 mg of polylysine (mol wt 75,000; degree of polymerization, 586) per ml of 12.5 mM phosphate buffer. The solution was adjusted to the desired pH as indicated by the numbers on the curves. The interaction time was 10 min, and the dithionite reduction time was 6 min.

Fig. 3. Absolute absorption spectra of the heme α-polylysine complex in alkaline medium. The final concentration was 1.5 μM poly-L-lysine and 11.6 μM hematin α at pH 10.6. Curves I, oxidized, as prepared; II, reduced with dithionite; III, reduced, subsequently saturated with carbon monoxide; IV, base line.
plex was also investigated. Addition of up to 8 mM cyanide to a solution of the complex at pH 11.6 did not shift the position of the absorption maxima, but caused a slight decrease in absorbance. Cyanide does not significantly affect the spectra of cytochrome oxidase.

Stoichiometry of Hematin α-Polylysine Complex—Spectrophotometric titration was used for the determination of the stoichiometry of the complex. Two samples of polylysine were used. When a constant amount of heme α was titrated with varying amounts of polylysine, the absorbance at 428 nm rapidly increased with the increase of the polyamino acid concentration. Fig. 5 shows the protocols of the titration. It was calculated from the curves that, at saturation, the ratio of heme α to polylysine is 46:1 for the sample of molecular weight 70,000, and 100:1 for that of 210,000. Since the average degrees of polymerization for these samples were 480 and 1,640, the ratios of lysine residues bound to heme α would thus be about 11 and 16, respectively. These figures are only approximate, to show the order of magnitude, because both the molecular weights and degrees of polymerization were merely taken from the manufacturer's label. Moreover, the molecular weights determined by physical methods, which were also supplied by the manufacturer, deviate somewhat with the values of the degrees of polymerization.

Interactions of Protohematin and Polylysine—Keilin (4) has suggested the interaction of protohematin and polylysine. Blauer (7, 12) and Blauer and Rottenberg (10) have described this reaction at different pH values for their physicochemical studies; however, they did not investigate the reduced form. Since protohematin does not possess a carbonyl group, it would be interesting to examine its behavior upon reduction by dithionite and hydrogenation by borohydride.

Protohematin reacted with polylysine in neutral as well as in alkaline media. The absorption spectra of the complex were dependent upon the age of the protohematin solution. The dithionite-reduced complex from the freshly prepared protohematin solution at pH 11.6 gave spectra with maxima at 556 to 558 nm and 432 nm; a distinct β-band at 528 nm was also discernible. The carbon monoxide compound showed maxima at 567 to 568 nm and 429 nm, with a shoulder at 541 nm. However, the complex formed from the aged protohematin exhibited not only different maxima but also a great decrease of over-all absorbance. Carbon monoxide reaction in the aged system caused a slight hyperchromicity, in contrast to the fresh protohematin solution, which showed a distinct hypochromicity (cf. Reference 33).

A comparison of some spectral characteristics between the polylysine complexes of heme α and protoheme and their derivatives at pH 11.6 is summarized in Table II. Significant differ-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>α-Band maximum</th>
<th>γ-Band maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A nm</td>
<td>B nm</td>
</tr>
<tr>
<td>1. None</td>
<td>556</td>
<td>432</td>
</tr>
<tr>
<td>2. NaSO₄</td>
<td>556</td>
<td>432</td>
</tr>
<tr>
<td>3. NaSO₄ + CO</td>
<td>567</td>
<td>429</td>
</tr>
<tr>
<td>4. Borohydride + NaSO₄</td>
<td>556</td>
<td>432</td>
</tr>
<tr>
<td>5. Borohydride + NaSO₄ + CO</td>
<td>567</td>
<td>429</td>
</tr>
</tbody>
</table>

* A, cytochrome oxidase; B, heme α-polylysine; C, heme α-globin; D, heme α-histone; E, protohematin-polylysine.
* For the oxidized form of heme α complexes and cytochrome oxidase, peaks in the α-region were very broad. The band width of the Soret maximum was, likewise, relatively broad.
* This maximum became 424 nm from the system with aged protohemine solution.
* This maximum became 418 nm from the system with aged protohemine solution.
* N.D., not determined.

hematin solution at pH 11.6 gave spectra with maxima at 556 to 558 nm and 432 nm; a distinct β-band at 528 nm was also discernible. The carbon monoxide compound showed maxima at 567 to 568 nm and 429 nm, with a shoulder at 541 nm. However, the complex formed from the aged protohematin exhibited not only different maxima but also a great decrease of over-all absorbance. Carbon monoxide reaction in the aged system caused a slight hyperchromicity, in contrast to the fresh protohematin solution, which showed a distinct hypochromicity (cf. Reference 33).
Enzyme activities clearly exist. The maxima of the reduced and carbon monoxide compounds of hemoglobin a complex were at longer wave lengths than those of the protoheme-polysine complex. In addition, the absorbance ratio of α- to β-maxima in the carbon monoxide compound of the heme a-polysine complex was greater than 1. This ratio was nearly or smaller than 1 in the carbon monoxide compound of the protobeme-polysine complex. When the formyl group either in free heme a or in the Schiff base complex with the peptide was hydrogenated by borohydride, the spectral difference between these complexes became much smaller, as seen in Systems 4 and 5 of Table II. At the same time, the absorbance ratio of α- to β-maxima for the carbon monoxide derivatives became nearly or smaller than 1, similar to the absorbance ratio of the carbon monoxide protobeme-polysine compound. This observation was expected; abolition of a chromophore such as a carbonyl group would cause a significant blue shift as a result of the change in electronic configuration of the heme. In turn, the perturbation effect of protein exerted on the heme, or the interaction with the heme, or both, might be different at different oxidation states of the heme.

Effect of Borohydride on Heme a-Polysine and Protobeme-Polysine Complexes — The formation of a Schiff base between the nonprotonated ε-amino group of lysine and the formyl group of hemoglobin a in either cytochrome oxidase or the polysine complex is only a postulate adduced from the spectral behavior of these compounds in alkaline media. In the case of cytochrome oxidase, the postulate has been greatly strengthened by the experiment with borohydride as reported previously (30). Borohydride hydrogenates the imine of the Schiff base to a more stable covalent bond, and, in turn, the heme a is “fixed” to the protein moiety and becomes no longer extractable with acid-acetone.

Experiments have confirmed this postulation (30). Therefore, this principle was applied to the study of the polysine complexes.

A solution of heme a-polysine complex at pH 11.6 was allowed to react with a slight excess of sodium borohydride for 3 hours at 0°C. Comparison of Fig. 3 with Fig. 6 reveals the blue shifts of both the α- and the γ-maxima due to the borohydride reaction. The dithionite-reduced form showed distinct maxima at 558 and 418 μm and a shoulder at 523 μm. The positions of these maxima are practically the same as those observed in cytochrome oxidase (30). Reaction with carbon monoxide further changed the maxima to 416 μm with broad peaks at 546 and 536 μm (Fig. 6).

Acid-acetone did not extract any significant amount of heme a from the heme a-polysine complex which had been subjected to borohydride reaction. Direct iron analysis was made in the acid-acetone fractions both the supernatant and the residue. Table III summarizes the results. The acid-acetone extract contained 25% of the iron originally present in the borohydride-reduced heme a-polysine complex, in contrast to extraction of about 86% of the heme without borohydride reduction. If, indeed, the “fixation” of the heme to the peptide is through the Schiff base, then the protobeme (which does not possess a carbonyl group) in the protobeme-polysine complex should be extractable with acid-acetone. Likewise, when hematin a is first reduced by borohydride to an alcohol derivative (cf. Ref.

![Graph](image-url)

**Fig. 6. Absolute spectra of the borohydride-treated heme a-polysine complex. Curve I, dithionite-reduced; II, dithionite-reduced and subsequently treated with carbon monoxide.**

**Table III**

**Direct iron analysis of acid-acetone extract and residue of some heme-protein complexes**

<table>
<thead>
<tr>
<th>System and fraction</th>
<th>Borohydride reduction</th>
<th>Distribution of iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Heme a-polysine</td>
<td>+</td>
<td>0.12</td>
</tr>
<tr>
<td>Acid-acetone extract</td>
<td>−</td>
<td>0.35</td>
</tr>
<tr>
<td>Residue</td>
<td>−</td>
<td>0.70</td>
</tr>
<tr>
<td>2. Heme a-polysine</td>
<td>−</td>
<td>0.07</td>
</tr>
<tr>
<td>Acid-acetone extract</td>
<td>+</td>
<td>0.38</td>
</tr>
<tr>
<td>Residue</td>
<td>+</td>
<td>0.15</td>
</tr>
<tr>
<td>3. Borohydride-reduced heme a-polysine</td>
<td>+</td>
<td>0.94</td>
</tr>
<tr>
<td>Acid-acetone extract</td>
<td>+</td>
<td>0.13</td>
</tr>
<tr>
<td>Residue</td>
<td>+</td>
<td>1.02</td>
</tr>
<tr>
<td>4. Protopheme-polysine</td>
<td>+</td>
<td>0.13</td>
</tr>
<tr>
<td>Acid-acetone extract</td>
<td>+</td>
<td>1.02</td>
</tr>
<tr>
<td>Residue</td>
<td>+</td>
<td>0.94</td>
</tr>
<tr>
<td>5. Heme a-globin</td>
<td>+</td>
<td>0.13</td>
</tr>
</tbody>
</table>
ence 38) prior to its reaction with the polypeptide, the heme a
in the polylysine complex should be extractable. Experiment-
ally, it was found as deduced. In both cases, the acid-acetone
extracts were highly colored. Chemical analysis for iron con
firmed the heme distributions, as also shown in Table III; 90 and
70% of the iron were recovered in the soluble extracts of the
protheme-polylysine complex and of the polylysine complex
with borohydride-pretreated heme a, respectively. Further-
more, the heme moiety of the borohydride-reduced complex of
heme a and globin (the latter isolated from bovine hemoglobin)
was also found to be nonextractable with acid-acetone.

These observations substantiate the claim that a Schiff base
is formed between the formyl group of heme a and the ε-amino
group of lysine in the synthetic polypeptide just as in cytochrome
oxidase. This Schiff base can be hydrogenated by borohydride
to a stable bond, so that heme a is fixed to the protein moiety and
becomes nonextractable by acid-acetone.

**Interactions of Hematin a and Globin**

The globin used (isolated from freshly prepared bovine hemo-
globin) was in the native state as ascertained by the recombi-
ation technique (34, 35). By spectrophotometric titration it was
found that the ratio of protohematin to globin was 4, in good
agreement with the value reported (35).

Globin reacted with hematin a in neutral media. The abso-
lute spectrum exhibited maxima at 593 to 595 μm and at 423 μm
as depicted in Fig. 7A. Upon reduction by dithionite, the
maxima shifted to 596 to 597 μm and 438 μm, with less prominent
peaks at 504 and 524 μm and a shoulder at 423 μm. Carbon
monoxide caused distinct spectral changes; maxima at 501 and
431 μm were observed. Morell et al. (17) have briefly reported
some spectral changes of hematin a attributable to the influence
of globin. Our results are essentially in agreement with their
data (17). In neutral media, borohydride altered the spectral
behavior of the heme a-globin complex. The maxima of the
borohydride-treated and subsequently dithionite-reduced form
became indistinct except for the Soret maximum at 421 μm.
Carbon monoxide treatment further shifted this band to 418 μm,
with a concurrent increase in absorbance (spectra not shown).

Globin reacted differently with hematin a in alkaline media.
At pH 11.6, the resultant globin complex exhibited an absorption
maximum at 408 μm and a broad a-band at approximately 630
μm, as shown in Fig. 7B. Upon reduction by dithionite, a dis-
tinct a-peak appeared at 575 μm, and a Soret band, at 428 to 430
μm. Carbon monoxide shifted the maxima to 586 and 424 μm,
with a shoulder at 542 μm. Borohydride reduction for 3 hours
at pH 11.6 changed the characteristics of the reduced spectrum,
with new maxima at 588 and 420 μm. After reaction with car-
bon monoxide, the borohydride-reduced complex showed bands
at 568 and 416 μm (spectra not shown). All these spectral
characteristics of the heme a-globin complex closely resembled
the behavior of heme a-polylysine as described in the previous
section.

Spectrophotometric titration for the reaction between hematin
a and globin in neutral medium gave a ratio between 4 and 5 moles (average, 4.7) of hematin per mole of the protein. At pH 11.6, this ratio was found to be approximately 14 (cf. Fig. 8). As judged from the titration curve in neutral medium, the binding constant for hematin a and globin must be smaller than that for protohematin. Consequently, the extrapolated value (4.7) is subject to a substantial error. However, at pH 11.6, the titration was relatively clear-cut; thus, the value found (13.8) was more reliable. At any rate, the reaction between hematin a and globin either at pH 7.0 or at pH 11.6 was slow; about 45 min were found necessary for complete reaction. This is in contrast to the recombination of protohematin and globin to hemoglobin, which has been found to be very rapid.

*Fixation of Hematin a to Protein by Borohydride Reduction of Hematin a-Globin Complex in Alkaline Media*—As recalled, in both cytochrome oxidase and the hematin a-polylysine complex, the imine of the Schiff base apparently formed can be hydrogenated by borohydride. In turn, the hematin group is fixed to the protein moiety and becomes no longer extractable with acid-acetone. On the other hand, the protohematin-polylysine complex does not share this property since protohematin has no carbonyl group. When the hematin a-globin complex was reduced by borohydride, it was found that the heme a was also fixed to the globin and could not be extracted by acid-acetone (Table III).

*Attempted Demonstration of Oxygenative and Oxidative Activity of Hematin a-Globin Complex*—The reversible oxygenation of hemoglobin is well known. The oxygenated complex of cytochrome oxidase has also been proposed (39), regardless of its nature and significance (40). We tested the oxygenative activity of the heme a-globin complex. No spectral difference was observed whether the reduced complex was oxidized by ferricyanide
or was saturated with oxygen. This fact seems to indicate that this hybrid molecule does not possess oxygenative activity or, at least, that this activity is not reflected in spectral behavior. The usual enzyme assay systems showed that the hematin a-globin complex to be devoid of either cytochrome oxidase or tetrahydroquinone oxidase activity. All these observations only suggest that the complex is autoxidizable from the ferrous to the ferric state, but that the ferric form cannot be reduced by reduced cytochrome c or by tetrahydroquinone. Details will be reported elsewhere.

**Interactions of Hematin a with Some Amino Acids, Polyamino Acids, and Proteins**

Hematin a did not react with L-histidine, L-lysine, L-glutamic acid, L-aspartic acid, L-proline, or L-alanine, in either neutral or alkaline media, under conditions similar to those used for polypeptide. Recently Yoshida, Kinoshita, Horie, and Schimazono (13) have reported the absorption spectra of heme a complexes with some amino acids, but their investigation was conducted in solutions containing 5 to 20% of amino acids, in contrast to the concentration of not more than 0.2% used in our study. However, the spectra of heme a in the presence of 5% lysine (but not other free amino acids tested, including also L-methionine, L-histidine, and L-tyrosine) at pH 11.6 closely resembled those of the heme a-polylysine complex at the same pH. This observation indicates that the binding constant of heme a with monomeric lysine is many orders smaller than that with polylysine. Absorption spectra of systems containing hematin a and high concentrations of histidine were found to be essentially the same as those reported previously (21, 22). These spectra are completely different from the spectra obtained in the system with lysine or polylysine, but are similar to those with polyhistidine (see below).

Most synthetic polyamino acids are insoluble in aqueous media. Several water-soluble polypeptides were tested under a restrictive condition. Polytotyrine (which is soluble only in alkaline solution) at pH 10 or 11.6, and polylglutamic acid and polyproline in neutral medium or at pH 11.6 apparently did not react with heme a; no distinct absorption maxima were observed under conditions similar to those in the systems used with polylysine.

Hematin a reacted with polysarcosine under the conditions detailed in the legend to Fig. 9. The absorption spectra of the oxidized and the reduced forms at pH 7.8 are presented in Fig. 9A. As shown, no distinct absorption maxima were observed under conditions similar to those in the systems used with polylysine. Hematin a reacted with polysarcosine under the conditions detailed in the legend to Fig. 9. The absorption spectra of the oxidized and the reduced forms at pH 7.8 are presented in Fig. 9A. As shown, no distinct absorption maxima were observed under conditions similar to those in the systems used with polylysine.
apparent fusion of two bands to give a relatively sharp maximum at 427 μm. These characteristics are quite different from those of the heme α-polylysine complex.

The behavior of polyhistidine toward hematin α was complicated; this polyamino acid is insoluble in aqueous media above pH 5.8 even in the absence of phosphate. At pH 5.7, the oxidized form of the complex showed absorption maxima at 594 and 423 μm. The spectrum remained practically the same even after standing for ½ hour. Upon dithionite reduction, turbidity developed, but the maxima were still distinguishable at 594, 562, and 423 μm, with a shoulder at approximately 442 μm, as shown in Fig. 10. The reduced form appeared to be rapidly autooxidizable. After adjustment of the system in the oxidized form to a pH higher than 6, a green precipitate formed. The precipitate was dissolved in dimethylsulfoxide and gave an apparent pH of approximately 8. The solution did not exhibit a distinct α-band in the oxidized form; by contrast, hematin α alone in dimethylsulfoxide showed maxima at 631 and 405 μm. Upon dithionite reduction in dimethylsulfoxide, hematin α showed distinct absorption maxima and the behavior of the borohydride reaction were the same as those with polylysine and globin. As shown in Fig. 11, the heme α-histone complex at pH 11.6 showed maxima at 574 and 428 μm in the dithionite-reduced form. Carbon monoxide treatment of the reduced complex shifted the maxima to 584 and 424 μm.

**DISCUSSION**

From the data presented here (cf. also References 16 and 17), together with the behavior of cytochrome oxidase described previously in publications by this laboratory (29, 30) and also by others (26, 28, 41), a number of salient points may be deduced. Here it suffices to point out a striking resemblance between the hematin α complexes of polylysine and of proteins with high lysine content, on one hand, and cytochrome oxidase in alkaline media, on the other. Spectral characteristics of these compounds, in comparison with the protohematin-polylysine complex and cytochrome oxidase, are summarized in Table II. Although absorbance indices differ, the positions of the band maxima of the hematin α complexes of albumin, globin, histone, and polylysine (as well as monomeric lysine at high concentrations) are practically the same as those of cytochrome oxidase at pH 11.6 in the oxidized, the reduced, the carbon monoxide-reduced, the borohydride-treated, and the borohydride-treated followed by carbon monoxide reduction forms. Moreover, after borohydride treatment the heme α in all these artificial complexes or linked hemoproteins, for example, might be interpreted as the difference of the ligands remains to be studied. At present, molecular spectroscopy is not advanced enough either to interpret meaningfully or to predict accurately the fine structure of hemoprotein spectra (cf. Section 2 of Reference 43); therefore other conceivable means must be pursued and explored.

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