Isolation and Properties of Two Soluble Heme Proteins in Extracts of the Photoanaerobe Chromatium*

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(Received for publication August 17, 1959)

The involvement of heme proteins in photometabolism, first suggested by Hill (1, 2), seems certain from results of recent enzyme studies (3-5) and observations on systems in vivo using differential spectrophotometry (6-10). Of the many heme proteins which exist in intimate association with the photoactive pigments in the functional subcellular structures of photosynthetic tissues, only three have been isolated in soluble form in sufficient amounts and purity for adequate characterization. One of these is the so-called "cytochrome f" of green plant tissues (11). The other two are variant cytochromes, one of the "c" type (12), and one an atypical heme protein called "RHP" (12, 13) obtained from extracts of the facultative phototrophic purple bacterium, Rhodospirillum rubrum. We now report the isolation and identification of two soluble heme proteins contained in extracts of the obligate photoanaerobic purple bacterium, Chromatium. Previous studies (14, 15) have indicated the existence of both a cytochrome "c"-type and an RHP-type heme protein in partially resolved preparations (15). Some preliminary data have been published on their physicochemical properties. Complete resolution of these heme protein mixtures has been achieved so that it is now possible to characterize completely the two soluble Chromatium cytochrome components.

METHODS

Bacterial Culture—Chromatium sp. strain D was grown in the following medium (all values are in grams per liter of tap water): NaCl, 10.0; K2HPO4, 1.0; (NH4)2SO4, 1.0; MgCl2·6H2O, 0.5; CaCl2, 0.05; NaHCO3, 2.0; FeCl3·7H2O, 0.005; and as oxidizable substrate either NaS·9H2O, 1.0, plus Na2S2O3·5H2O, 2.0, or hydrogen gas which was bubbled continuously through the culture; pH 8. The methods followed for growth, collection and storage of the organism were as described by Newton and Kamen (15).

Extraction of Heme Proteins—As much as one-half of the heme protein eventually extracted from the bacteria was obtained by freezing and thawing the cells, suspending the thawed cell mass in an equal volume of 0.05 M phosphate buffer, pH 7, and centrifuging the suspension for 10 minutes at 25,000 × g. Additional material was extracted by suspending the residue in 1.5 volumes phosphate buffer and treating the suspension for 5 to 10 minutes in a 10 kc Raytheon sonic oscillator. The suspension was centrifuged for 10 minutes at 25,000 × g to remove cell debris. This sediment was resuspended and extracted as before. All extracts were combined and 35 g of ammonium sulfate were added to each 100 ml of extract. The suspension was left in the cold overnight and was then centrifuged for 30 minutes at 25,000 × g to remove the voluminous precipitate which consisted mainly of subcellular particles. The precipitate was resuspended in 2 volumes of a solution containing 35 g of ammonium sulfate per 100 ml of water. After standing in the cold several hours, the suspension was centrifuged as before. This washing procedure was repeated twice more.

The extracts were combined and 25 g of additional ammonium sulfate were added to each 100 ml of solution to precipitate the heme proteins. The precipitate was separated by centrifugation, and was dissolved in a minimal amount of water to which solid Tris was added to adjust the pH to 7.5 and passed through a 8-cm DEAE-cellulose column. The unadsorbed protein solution was completely displaced from the column by 0.02 M Tris, pH 7.6. Contaminating nucleotide material which contributed to the large light absorption at 260 mμ was removed by washing the column with several changes of 0.02 M Tris, pH 7.6. The amount of DEAE-cellulose required to adsorb the protein eventually extracted from the bacteria was obtained by freezing and thawing the cells, suspending the thawed cell mass in an equal volume of 0.05 M phosphate buffer, pH 7, and centrifuging the suspension for 10 minutes at 25,000 × g. Additional material was extracted by suspending the residue in 1.5 volumes phosphate buffer and treating the suspension for 5 to 10 minutes in a 10 kc Raytheon sonic oscillator. The suspension was centrifuged for 10 minutes at 25,000 × g to remove cell debris. This sediment was resuspended and extracted as before. All extracts were combined and 35 g of ammonium sulfate were added to each 100 ml of extract. The suspension was left in the cold overnight and was then centrifuged for 30 minutes at 25,000 × g to remove the voluminous precipitate which consisted mainly of subcellular particles. The precipitate was resuspended in 2 volumes of a solution containing 35 g of ammonium sulfate per 100 ml of water. After standing in the cold several hours, the suspension was centrifuged as before. This washing procedure was repeated twice more.

The extracts were combined and 25 g of additional ammonium sulfate were added to each 100 ml of solution to precipitate the heme proteins. The precipitate was separated by centrifugation, and was dissolved in a minimal amount of water to which solid Tris was added to adjust the pH to 7.5. The crude heme protein extract was dialyzed against several changes of distilled water until free of salts.

Next, the extract either was chromatographed directly on a large DEAE-cellulose column or, as exemplified in Table I, was passed first through a DEAE-cellulose column to remove nucleotide material which contributed to the large light absorption at 260 mμ. The amount of DEAE-cellulose required to adsorb the nucleotides was determined by preliminary titration of an aliquot of the extract. The main portion of the extract was then adjusted to pH 7.5 and passed through a 4 × 8 cm DEAE-cellulose column. The adsorbed protein solution was completely displaced from the column by 0.02 M Tris, pH 7.6. Contaminating red colored particulate material (small particles) as well as nucleotide material remained on the column. The nucleotide-free protein solution was dialyzed against 20 volumes of the Tris buffer preparatory to chromatography on a DEAE-cellulose column.

Chromatographic Separation and Purification of Chromatium Cytochrome c and RHP—The cytochrome c and RHP were separated and purified by two successive chromatographic steps on
to rechromatography. At this stage the two heme protein frac-

tions were free of each other, but each was contaminated by extraneous nonheme protein.

The two fractions were rechromatographed on separate 2- × 20-cm DEAE-cellulose columns by the above procedure. The purest portions of the main fractions were pooled, concentrated, dialyzed until free of salt, and finally lyophilized. By the criteria described below the best preparations were essentially homog-

geneous.

The use of DEAE-cellulose columns as described was also found applicable to the isolation and purification of the RHP and cytochrome c components of R. rubrum extracts and represents a considerable simplification over the isoelectric precipitation method reported by us (13).

**Estimation of Heme Protein Concentrations**—The concentrations of RHP and cytochrome c in various extracts were estimated from the optical densities of absorption bands characteristic for each heme protein in reduced-minus-oxidized difference spectra, using the extinction coefficients recorded in Table V. For RHP, the hematin band at 635 mp, and, for cytochrome c, the 3 band at 524 mp, were established as wave lengths at which neither component contributed significantly to the optical density of the other. The difference spectra were measured with a Cary model 14 spectrophotometer equipped with a 0.1 optical density full scale slide wire.

**Physical Measurements**—Sedimentation constant and electrophoretic mobility determinations were performed for us by Dr. Karl Schmid, Massachusetts General Hospital, using the Spinco analytical centrifuge and Perkin Elmer electrophoresis appara-
tus, respectively. The methods employed are identical with those described previously (13). The diffusion constant D was determined for us by Dr. Lawrence Levine, Brandeis University, using the Oudin gel diffusion procedure (17). Partial specific volume values were determined with 5-ml pycnometers (18).

Oxidation-reduction titrations were performed by the method of Hill (19) employing the ferric-ferrous oxalate system (13).

**Preparation of Antisera to the Purified Cytochromes**—Antisera were produced in rabbits by the intravenous injection of alum-

precipitated cytochromes. Sixteen injections were given, one every other day, the doses being increased gradually from 0.5 to 7.5 mg of protein. The rabbits were bled 6 days after the last injection. The sera were cleared by centrifugation and complement (C') was inactivated by heating for 30 minutes at 56°.

**RESULTS**

**Physical and Chemical Properties of Chromatium Heme Proteins**—A number of physical and chemical properties of Chromatium cytochrome c and RHP are summarized in Table III. Analogous data for R. rubrum heme proteins are included for comparison.

The spectrochemical behavior of the Chromatium RHP, when subjected to varying pH and treated with ligand-donating re-

agents, was found to be identical with that of the corresponding heme protein extracted from R. rubrum (13). The Chromatium cytochrome c exhibited the general chemical characteristics of autoxidizable c-type cytochromes, such as those reported to occur in extracts of various anaerobic bacteria (20, 21).

The two heme proteins were acidic, with isoelectric points close to pH 5.5, as determined by interpolation of mobility values (Table IV). Molecular weights calculated from S, D, and V values for RHP and cytochrome c were 36,000 and 97,000, respectively.

**TABLE I**

**Extraction of Chromatium heme proteins**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Optical density ratio 280/260</th>
<th>Protein*</th>
<th>Cyto-</th>
<th>RHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract* (dialyzed ammonium sulfate precipitate)</td>
<td>150</td>
<td>0.65</td>
<td>s</td>
<td>0.59</td>
<td>0.83</td>
</tr>
<tr>
<td>Nucleotide-free extract</td>
<td>135</td>
<td>1.2</td>
<td>5.75</td>
<td>0.54</td>
<td>0.67</td>
</tr>
</tbody>
</table>

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* The protein concentration was estimated from the optical densities at 280 and 260 mp.

b The crude extract was prepared from 600 g (wet weight) of hydrogen-grown Chromatium cells.

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**TABLE II**

**Chromatographic purification of Chromatium cytochromes**

<table>
<thead>
<tr>
<th>RHP fraction</th>
<th>Protein</th>
<th>Optical density ratio Soret/280</th>
<th>RHP</th>
<th>Protein</th>
<th>Optical density ratio Soret/280</th>
<th>Cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st chromatogram on DEAE-cellulose column</td>
<td>0.58*</td>
<td>0.45</td>
<td>0.34</td>
<td>2.06*</td>
<td>0.65</td>
<td>0.82</td>
</tr>
<tr>
<td>2nd chromatogram on DEAE-cellulose column</td>
<td>0.090*</td>
<td>0.33</td>
<td>0.090</td>
<td>0.397*</td>
<td>0.53</td>
<td>0.397</td>
</tr>
</tbody>
</table>

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* Protein weight estimated from optical densities at 280 and 260 mp.

b Dry weight values for the lyophilized pure preparations.

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DEAE-cellulose columns by the general methods of Sober et al. (16). The results are summarized in Table II.

The nucleotide-free protein solution was first chromatographed on a 4.0- × 20-cm DEAE-cellulose column which had been equili-

brated with 0.02 M NaCl. After the protein solution had been applied to the column it was washed with the same buffer; the ionic strength was increased stepwise by the addition of so-

dium chloride in 0.03 M increments for every 300 to 400 ml of solution that passed through the column until a salt concentra-

tion was reached at which a colored protein band began to move. The mobile band was then eluted at a slightly (0.01 to 0.02 M) higher salt concentration. The cluters were collected in tubes, using a fraction collector. The ratio of the protein to Soret band optical density values was used as an index of purity for deter-

mining which fractions of a given colored band were to be pooled. Three main fractions were eluted: a green colored fraction by 0.04 to 0.05 M NaCl, a brown colored fraction (RHP) by 0.07 to 0.1 M NaCl, and a red-brown fraction (cytochrome c) by 0.13 to 0.15 M NaCl.

The pooled fractions were concentrated to about 0.1 vol-

ume by lyophilization. The concentrated samples were dialyzed against several changes of distilled water until free of salts and then were equilibrated against 0.02 M Tris, pH 7.6, preparatory to rechromatography. At this stage the two heme protein frac-

tions were free of each other, but each was contaminated by extraneous nonheme protein.

The two fractions were rechromatographed on separate 2- × 20-cm DEAE-cellulose columns by the above procedure. The purest portions of the main fractions were pooled, concentrated, dialyzed until free of salt, and finally lyophilized. By the criteria described below the best preparations were essentially homo-

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The two heme proteins were acidic, with isoelectric points close to pH 5.5, as determined by interpolation of mobility values (Table IV). Molecular weights calculated from S, D, and V values for RHP and cytochrome c were 36,000 and 97,000, respectively.

The heme contents of these cytochromes were de-
determined from alkaline pyridine hemochromogen spectra (22). If only one heme per molecule was assumed, the molecular weights of RHP and cytochrome c were calculated to be 17,000 and 37,000, respectively. These results indicated that Chromatium RHP might contain two heme groups per molecule and the cytochrome c might contain as many as three hemes per molecule. No evidence was obtained in sedimentation runs at varying concentrations to support the possibility that dissociable aggregates of the heme proteins were present.

Neither of the two heme proteins proved readily amenable to iron content determinations by the usual colorimetric procedures (22, 23) after wet ashing. This experience was much like that noted previously with the RHP of <i>R. rubrum</i> (13). When it became evident that further efforts to define proper ashing conditions might prove prohibitively wasteful of material, we deferred determinations of iron content to a future time when sufficient pure protein could be accumulated.

As measured by titration with the ferri-ferrous oxalate oxidation-reduction system, both heme proteins had relatively low standard potential values: <i>E</i><sub><i>r</i></sub> was -0.005 volt for RHP and +0.01 volt for the cytochrome c. As expected of heme compounds with such relatively low oxidation-reduction potentials, both proteins were autoxidizable.

**Immunochemical Properties of Chromatium Heme Proteins**—Immunochemical properties of the Chromatium cytochromes are being studied in collaboration with Dr. L. Levine and will be reported in greater detail elsewhere. However, some preliminary evidence bearing on the purity of the heme proteins is noted here.

Antisera to the purified proteins and to a crude Chromatium sonic extract completely freed of bacterial particles by centrifugation, as described by Newton and Newton (24), have been used as test systems. In qualitative Ouchterlony gel diffusion tests (25) only one precipitation band was formed between an antigenic heme protein and the homologous antiserum. The heme moiety of each antigenic protein was recovered quantitatively in the washed precipitate obtained when antigen was treated with excess antibody. No cross reaction between one heme protein and the antiserum to the other heme protein occurred. When tested against the crude extract antiserum, each heme protein gave a single characteristic precipitation band. At least 7 precipitation bands were found when crude extract was reacted with the crude extract antiserum. These observations indicated that the heme proteins were not contaminated one with the other nor with other antigenic proteins derived from the bacterial extract.

Part of one cytochrome c sample which was accidentally denatured by being exposed to approximately pH 12 for some time was found to behave differently from samples which had not been so treated (see also discussion below of cytochrome c reaction with carbon monoxide). In the Ouchterlony test this cytochrome c sample gave no precipitation band when tested at several concentrations with anticytochrome c. When normal and alkaline cytochrome c samples were tested side by side for reaction with crude extract antiserum on the same Ouchterlony plate, reactions of partial identity were observed (25), indicating similarity between the two antigens. It was found by immunochromical techniques (26) that the alkali-treated sample inhibited the reaction between the homologous cytochrome c and the antibody, indicating that the two cytochrome samples were structurally similar. It was tentatively concluded that elevated pH denatured the cytochrome c to such an extent as to change but not completely abolish the antigenic properties of the protein. As mentioned below, the reactivity of the cytochrome c toward carbon monoxide may be related to denaturation of the heme protein.

**Spectroscopic Observations**—Extinction coefficients and absorption peaks for both RHP and cytochrome c are tabulated in Table II. The spectra of Chromatium RHP resembled closely those previously reported for <i>R. rubrum</i> RHP (13). Reduced Chromatium RHP had a three-banded hemochromogen type spectrum with the Soret band at 426 mμ and with <i>α</i> and <i>β</i> peaks so closely spaced as to appear fused in spectrophotometer tracings (Fig. 1). However, with a Hartridge reversion spectroscope the two bands were still plainly distinguished at 566 and 547 mμ, respectively. Similarly, <i>R. rubrum</i> RHP showed <i>α</i> and <i>β</i> absorption bands at 566 and 550 mμ so closely spaced as to appear as a fused band in a spectrophotometer tracing (13). The so-called "hematin"
TABLE V
Extinction values* of Chromatium heme proteins

<table>
<thead>
<tr>
<th>State of cytochrome</th>
<th>Cytochrome c</th>
<th>RHP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wave length of absorption peak (d = 1 cm)</td>
<td>cm/m</td>
</tr>
<tr>
<td>Oxidized</td>
<td>278</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>410</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>525</td>
<td>26</td>
</tr>
<tr>
<td>Reduced</td>
<td>416</td>
<td>364</td>
</tr>
<tr>
<td></td>
<td>523</td>
<td>41.6</td>
</tr>
<tr>
<td></td>
<td>559</td>
<td>61.1</td>
</tr>
<tr>
<td>Difference spectrum (reduced minus oxidized)</td>
<td>406</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>422</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>504</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>553</td>
<td>44.6</td>
</tr>
<tr>
<td>Carbon monoxide complex (reduced)</td>
<td>414</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>533</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td>555</td>
<td>50.5</td>
</tr>
<tr>
<td>Difference spectrum (reduced carbon monoxide minus reduced)</td>
<td>414</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>535</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>565</td>
<td>17.5</td>
</tr>
</tbody>
</table>

* The spectra were measured in pH 6.8, 0.05 M phosphate buffer; d = 1.0 cm, using the Cary model 14 spectrophotometer.

band, characteristic of the oxidized R. rubrum heme protein with an absorption maximum at 635 μm, also appeared in the spectrum of oxidized Chromatium RHP.

Reduced Chromatium RHP reacted with carbon monoxide at neutral pH to give a three-banded hemochromogen spectrum with absorption peaks at 563, 535, and 418 μm. This behavior contrasted with that of the R. rubrum heme protein which, although forming a carbon monoxide complex at pH 7, only showed the definite α and β bands of a carbon monoxide hemochromogen at pH 5 or less. The influence of pH on Chromatium RHP carbon monoxide complex spectra has not been investigated.

The Soret band of the Chromatium RHP carbon monoxide complex was intensified about 2.5 times as compared with the reduced RHP band. As indicated by partial reversion of the α and β bands to the normal reduced spectrum intensities, the carbon monoxide complex of RHP at neutral pH under 100 per cent carbon monoxide was approximately 50 per cent dissociated when the sample was illuminated in the spectrophotometer by an approximately 400 foot-candle beam of white light. In the dark the spectrum of the carbon monoxide complex re-formed completely. The kinetics of this reversible dissociation were not investigated.

The locations and intensities of reduced-minus-oxidized and of the carbon monoxide-reduced-minus-reduced difference spectra of Chromatium RHP are summarized in Table V.

Chromatium cytochrome c exhibited a typical c-type hemochromogen three-banded spectrum with peaks at 552, 523, and 416 μm, as previously reported (15).

The cytochrome c reacted with carbon monoxide at neutral pH. The change in spectrum that occurred when 100 per cent carbon monoxide replaced a helium atmosphere over reduced cytochrome c in a sealed cuvette is illustrated in Fig. 2. Secondary α and β absorption bands at 563 and 533 μm appeared as shoulders on the remnants of the α and β bands of the reduced pigment and the Soret band at 414 μm was intensified. Extinction values for the various absorption bands of the complex formed by the particular sample used for Fig. 2 are recorded in Table V. However, the relative intensities of the two sets of α and β bands varied with the cytochrome sample used so that the numerical values were of little significance. The alkali-denatured cytochrome c sample mentioned earlier showed only the 563 and 533 μm peaks. The carbon monoxide complexes formed by the cytochrome c samples tested were all reversibly dissociated when illuminated by white light, as described in connection with the RHP-carbon monoxide complex.

The cytochrome c carbon monoxide-reduced-minus-reduced difference spectrum was hardly distinguishable from the difference spectrum of the RHP-carbon monoxide complex.

Apparently one or more of the heme groups of the purified cytochrome c can react with carbon monoxide and all the heme groups of the alkali denatured cytochrome are reactive. Perhaps the partial reaction between carbon monoxide and the heme iron is the consequence of partial denaturation of the cytochrome c. However, neither 5 × 10^-3 M potassium cyanide nor 5 × 10^-8 M potassium fluoride gave a spectroscopically detectable reaction with oxidized Chromatium cytochrome c or RHP at neutral pH, indicating that any denaturation that might have occurred was insufficient to expose heme groups to reaction with these negatively charged ligands.

![Fig. 1. Spectra of oxidized and reduced Chromatium RHP at pH 6.8.](http://www.jbc.org/)
In the past, it was apparent that the component responsible for the oxidative function of this protein in the bacterial cell. However, this protein could react with carbon monoxide only after denaturation then sonic extraction treatment caused the denaturation of the particle-bound as well as the soluble cytochrome c heme protein.

**DISCUSSION**

Preparations of heme proteins obtained from *Chromatium* in earlier researches (15) were highly acid and alkali labile and could neither be purified satisfactorily nor characterized completely except as a mixture of two types of heme protein, one being a cytochrome of the "c" type and the other resembling the cytochrome c variant, or RHP. It was concluded that probably only *Chromatium* cytochrome c remained in the particles, and that if this heme protein could react with carbon monoxide only after denaturation then sonic extraction treatment caused the denaturation of the particle-bound as well as the soluble cytochrome c heme protein.

The heme group of RHP has not been characterized by unequivocal means. The reduced alkaline pyridine hemochromogen spectra of *Chromatium* RHP and cytochrome c and of mammalian cytochrome c are identical in all details except molar extinction values, indicating that the heme groups of these three proteins are closely similar, if not identical.

**Heme Proteins in Chromatium Particles—**Newton and Newton (24) found that *Chromatium* particles contained a relatively large amount of heme protein as judged by the quantity of pyridine hemochromogen released from particles from which the photosynthetic pigments had first been extracted with organic solvents. We attempted to determine directly the kind of particle-bound heme proteins present in *Chromatium* washed particles prepared by sonic extraction of whole cells. The reduced-minus-oxidized difference spectrum of a particle suspension showed absorption peaks at 553, 524, and 422 mp, identical with those of the soluble cytochrome c. No indication of a hematin peak at 635 mp was found even at the highest practicable particle concentration, indicating that no readily detectable amount of RHP was present. A carbon monoxide-reduced-minus-reduced difference spectrum was also obtained with the particles, but because both cytochrome c and RHP give nearly indistinguishable carbon monoxide difference spectra, the heme protein (or proteins) present in the particles could not be identified by this method. In preliminary experiments no immunochemical reaction was detected between the particles and either anticytochrome c or anti-RHP. It was concluded that probably only *Chromatium* cytochrome c remained in the particles, and that this heme protein could react with carbon monoxide only after denaturation then sonic extraction treatment caused the denaturation of the particle-bound as well as the soluble cytochrome c heme protein.

**FIG. 2. Spectra of reduced and carbon monoxide-reduced Chromatium cytochrome c at pH 6.8.**

For the lability observed in the earlier preparations is attributable to the cytochrome c, which perhaps is still obtained in a partly denatured form, as evidenced by its variable reactivity with carbon monoxide. In its physical properties (high molecular weight, multiple heme composition, spectroscopic constants) it resembles the green plant cytochrome c ("cytochrome f") (11), but differs from cytochrome f in having a low oxidation potential ($E^\circ = 0.01$ volt). The true oxidation potential may be different from this observed value if the protein is partly denatured. It is premature to speculate on the oxidation-reduction function of this protein in the bacterial cell. However, this cytochrome is relatively easy to isolate and purify, and it affords one of the few c-type cytochromes obtained with relative ease from a strict anaerobe. Others are the cytochrome c of *Desulfobacterium* (31, 32) and the c-type cytochromes of the green sulfur photosynthetic anaerobes (33). These are all valuable test objects for extension of comparative studies on the heme peptide which appears to be characteristic of all c-type cytochromes (34). Although the prosthetic group of the *Chromatium* cytochrome c is linked in a manner identical with that of other cytochromes of the c (hemeoheme)-type, the Soret peak of its carbon mon-
The RHP-type protein is of special interest because it is only the second example of such a heme protein to be obtained in quantity and with a high degree of purity. The first example, or prototype, is that obtained originally from R. rubrum and described in detail by us in a previous paper (12). Other examples have been isolated in crude form from all known species of the purple photosynthetic bacteria (30) but do not appear to occur in the green photosynthetic sulfur bacteria (M. D. Kamen, unpublished observations). This failure to find RHP is no longer surprising, inasmuch as the green sulfur bacteria also differ radically from the purple bacteria in the nature of their photoactive pigments (35).

There is no question that on chemical and spectroscopic grounds the Chromatium RHP can be assigned to the same group of heme proteins as the RHP of R. rubrum and the other Allothrombodeaceae. Examination of Table III shows that differences in physical properties between the two types of RHP (other than possibly the heme composition and molecular weight) are no more variable than those encountered in different cytochromes of the c type. In this connection, we may note the wide divergences between physical properties of the c cytochromes obtained from Chromatium and R. rubrum (Table III). This point has been elaborated elsewhere (21) for all of the cytochromes of the c type.

A difficulty arises, however, in assigning RHP-type proteins to the same group classification when function is considered. Evidence has been presented implicating the R. rubrum protein in an oxidase system (13). Chance and Smith (8) and Smith and Ramirez (10) have provided evidence from their studies on changes in steady-state optical absorption of metabolizing R. rubrum suspensions that appears to be consistent with a terminal oxidase function for RHP. However, such a conclusion cannot be applied to the Chromatium RHP because Chromatium is a strict anaerobe and cannot couple reduction of molecular oxygen to useful metabolism. Indeed, oxygen inhibits the photosynthesis of ATP by Chromatium chromatophores, as does all the photosphorylation systems obtained from the photosynthetic tissues studied to date (36–39). A pseudo-oxidase activity can often be observed in extracts of anaerobes, arising from the presence of autoxidizable components such as flavins, —HS enzymes, and low oxidation-reduction potential cytochromes; this sort of "oxidase" is not to be confused with either the cytochrome a type of oxidase, found in aerobes, or the other types of oxidase noted in facultative anaerobes, such as the Allothrombodeaceae, the nitrate reducers, etc.

The Chromatium RHP resembles closely one of the active heme components observed by Olson and Chance in their extensive studies of the rapid transient changes in optical density brought about when actively metabolizing Chromatium suspensions were subjected to a variety of environmental conditions with and without illumination (40, 41). Another component they observed is a c-type cytochrome which may conceivably be the native form of the cytochrome c we have isolated. There seems to be little doubt that the RHP protein as well as a cytochrome c component are linked in some way to the photometabolism of Chromatium.

Olson and Chance have suggested that at least four distinct heme-containing proteins are involved in the transitions from light to dark metabolism of Chromatium. We have been able to observe only two heme proteins which are capable of being solubilized and although others may be present in firmly bound particle form, this possibility cannot be examined using isolation procedures commonly employed hitherto. It should be noted that a total of as many as five distinct heme groups may be present in the two proteins isolated. It is conceivable that these heme groups represent single heme proteins which have become firmly associated.

Based on pyridine hemochromogen yields obtained by treatment of whole cells with strong alkali and pyridine (15), it is estimated that perhaps 3 to 5 times more heme protein is present in the intact Chromatium cell than is obtained in the form of soluble RHP and cytochrome c. The Chromatium cell residues produced with our present methods exhibit difference spectra characteristic only of the soluble cytochrome c.

It is proposed in future work to attempt the isolation of heme proteins using the more subtle procedures now available based on preliminary treatment with agents such as Versene and lysomyze which render the cells osmotically unstable. Further, it seems possible to apply immunological procedures to monitor possible denaturation during extraction and purification. Also, the nature and function of the green pigment obtained as a third protein component remains to be determined.

Finally, it is perhaps warranted to comment on the suggestion that there exists a general class of heme proteins of which "RHP" is a prototype (21, 42). No examples of this kind of heme protein have been isolated and purified other than those obtained from the purple photosynthetic bacteria. The possibility that many such proteins can exist may be inferred from the presence in a wide variety of aerobes and facultative bacteria of carbon monoxide-binding pigments which appear to function as oxidases and which have spectroscopic properties much like those of the bacterial RHP forms studied in R. rubrum and Chromatium (43). Chance et al. (44) and Castor and Chance (45) have applied the tentative appellation "cytochrome O" to these proteins and surmised that they may be identical with RHP. Until one or more of these spectroscopic entities has been obtained as a pure protein and its properties determined, it seems premature to postulate a new general class of heme proteins. It may be noted, nevertheless, that one apparently authentic RHP-type protein, partially purified, has been described by Kono et al. (46) and Taniguchi et al. (47) as part of a hydroxyamine-reductase enzyme occurring in a "halo-tolerant" aerobe. It is possible that the realization that such heme proteins exist may spur investigators to search for them in many varieties of tissue, utilizing the present knowledge of the properties of RHP as exemplified in the examples obtained from the photosynthetic bacteria.

**SUMMARY**

1. The soluble heme proteins obtained from the obligate phototrophic Chromatium by various extraction procedures have been resolved into two homogeneous components. One component is a c-type cytochrome, the other is a variant heme protein closely similar to the RHP-type previously found in extracts of Rhodospirillum rubrum, a facultative photobacterote.

2. Procedures for purification based on DEAE-cellulose column chromatography and characterization of the soluble Chromatium heme proteins are described.

3. The physical and chemical properties of highly purified...
samples of these heme proteins are described. The RHP-type protein has an isoelectric point at pH 5.5 and appears to have two heme prosthetic groups per molecule. The molecular weight is 36,000. The cytochrome c is also an acidic protein with isoelectric point at pH 5.4. Its molecular weight is 97,000 and there appear to be three heme groups associated with each molecule.

4. Immunochemical investigations show the RHP and cytochrome c components to be antigenically different.

5. The possible functions of the two heme proteins in the metabolism of Chromatium are discussed, as well as the significance of present findings for continued studies on the relation of structure and function in heme proteins.

REFERENCES
