BBA 46197

IRON-CONTAINING PROTEINS IN *CHROMATIUM*

II. PURIFICATION AND PROPERTIES OF CHOLATE-SOLUBILIZED CYTOCHROME COMPLEX

S. J. KENNEL AND M. D. KAMEN

Department of Chemistry, University of California, San Diego, La Jolla, Calif. 92073 (U.S.A.)

(Received May 24th, 1971)

SUMMARY

A cytochrome complex was solubilized from the membrane fraction of *Chromatium* and purified by chromatography on hydroxylapatite. The purified cytochrome contained two different redox potential heme moieties: *c*-556 (+325 mV), and *c*-552.5 (+8 mV). The detergent-solubilized cytochrome was found to be identical to the membrane-bound form with respect to spectra, redox potentials, and absence of CO-binding capacity. Assuming a ratio of 1 *c*-556:2 *c*-552.5 in the complex, the reduced–oxidized absorption constants for the two spectral forms are: high potential cytochrome, ε_{red-ox} (556 nm) = 15.1 mM^{-1} and ε_{red-ox} (422 nm) = 103 mM^{-1}; low potential cytochrome, ε_{red-ox} (552.5 nm) = 13.1 mM^{-1} and ε_{red-ox} (423.5 nm) = 89 mM^{-1}. The spectral and chemical properties of the cytochrome complex show that none of the buffer-soluble cytochromes can be identified with this cytochrome complex.

INTRODUCTION

Light-induced cytochrome oxidation in *Chromatium vinosum* stain D has been studied for some time[1–5]. It has been assumed that the cytochromes involved are membrane-bound forms of the soluble cytochromes[6,7]. As described in the preceding article[8], it has been established that membrane-bound cytochrome is the predominant species in *Chromatium*. Furthermore, the membrane cytochrome can be solubilized with cholate. This report describes the purification and characterization of this cholate-soluble cytochrome complex, and presents evidence indicating that it cannot be related to the buffer-soluble cytochromes.

METHODS

Cell culture and cytochrome extraction

Cells were cultured in the malate medium, broken, and fractionated, as described previously[8]. Solubilization of cholate cytochrome complex was as reported[8], except that instead of crude chromatophores, the starting material used was the subchromatophore particle Fraction A, as isolated by THORNBER[9].

Abbreviation: PMS, phenazine methosulfate.

Hydroxylapatite preparation and cytochrome purification

Hydroxylapatite was prepared by a modification of the method of SIEGELMAN *et al.*[10]. Columns were poured from a thick, uniform slurry of hydroxylapatite in 0.2 M NaCl *plus* 0.01 M phosphate, pH 7.0. The crystals were permitted to settle before starting the column flow. It was observed that columns of length more than twice the breadth produced tight packing and crystal breakage with resultant slow flow rates. This also resulted if buffer pressure heads greater than the length of the column were applied.

For purification of the *Chromatium* cholate-soluble cytochrome, the crude cholate extract applied to the column contained 2 % cholate, 0.2 M NaCl, 0.1 % β-mercaptoethanol, and 50 mM Tris, pH 8.0. The major portion of the cytochrome formed a visible colored band which moved slowly down the column in this buffer concentration. Depending on the batch of hydroxylapatite, the cytochrome moved through about one bed volume of adsorbent for each 2–3 bed vol. of buffer. The protein was loaded onto the column until the colored front band was 1/2–2/3 of the way down the column. The column was then washed with the buffer solution until the cytochrome band was eluted. The column was then stripped of remaining cytochrome with a mixture containing 2 % cholate, 0.2 M NaCl, 0.2 M sodium phosphate, pH 7.0. After desalting on Sephadex G-25, this fraction could be rechromatographed by the above method to yield more purified cytochrome.

Techniques for protein analysis

The amino-terminal amino acids of the cholate-soluble cytochrome complex were identified by the EDMAN[11] degradation procedure as modified by DOOLITTLE[12]. Phenylthiohydantoin amino acids were hydrolyzed in evacuated tubes at 150° for 24 h. For amino acid composition studies, protein hydrolyses were conducted in evacuated tubes at 110° for 24, 48, or 72 h. Free amino acids from hydrolyzed proteins were analyzed on a modified Spinco automatic amino acid analyzer by the method of DUS *et al.*[13,14]. The yields of labile amino acids, such as threonine and serine, were determined by extrapolating the values observed to zero hydrolysis time.

Hydrazinolysis for carboxyl terminal determination was performed according to the method of DUS *et al.*[15] except that the hydrazides were removed from the free amino acids by chromatography on Amberlite IRC-50 ion exchange resin (Mallinkrodt). The hydrazinolysis mixture was passed through a 0.9 cm × 3 cm column of the resin in the H^+ form at pH 4–5. The acidic amino acids did not adsorb on the column and the basic and neutral amino acids were eluted with 0.3 M pyridine acetate, pH 7.0. The method gave poor recovery of lysine, arginine and tryptophan.

The lipid contents of the *Chromatium* cholate cytochrome complex and of the subchromatophore Fraction A and Fraction B[9] were determined by thin layer chromatography in diisobutyl ketone–acetic acid–water–ethanol, (80:50:10:10, v/v/v/v)[16]. The dried plate was first stained with I_2 vapor and then sprayed for phospholipid staining[16] with phosphomolybdate.

Spectroscopy

Absorption spectra were recorded using a Cary Model 14R recording spectrophotometer equipped with a combination 0–1.0, 2.0 and 0–0.1, 0.2 slide wire. All spectra were measured at pH 7.0 in 0.1 M potassium phosphate, unless otherwise

specified. The cytochrome was oxidized by passage through a small column of Amberlite IRC 400 ion exchange resin approximately half saturated with $K_3Fe(CN)_6$.

Spectra of the *Chromatium* cholate soluble cytochrome at 77°K were measured by Mr. David Hopkins using a modified Cary 14 spectrophotometer. The photomultiplier signal was analyzed by an analog digital converter (PDP 8/I computer, Digital Equipment Corp., Maynard, Mass.)[17].

Circular dichroism spectra were recorded by Mr. Michael Glaser on a Durrum-Jasco Model J-10 spectropolarimeter (distributed by Durrum Instrument Corp., Palo Alto, Calif.).

Chemical redox titrations

Chemical redox titrations were conducted in the cuvette and electrode assembly of CUSANOVICH AND BARTSCH[7]. An expanded scale pH meter (IL Model 145) was used to measure the redox potentials. The small sintered glass frit of the calomel reference electrode was replaced with a porous Teflon plug (5 μm pore size, 55 % air by vol., Fluoro-Plastics, Inc., Philadelphia, Pa.) to facilitate rapid equilibration with redox mediators after the original porous plug became clogged in use. The combination Pt–calomel electrode assembly (IL 15020) was standardized by titration of $K_3Fe(CN)_6$ ($E_{m,7} = 430$ mV)[18], toluylene blue ($E_{m,7} = +115$ mV)[18], indigotetrasulfonic acid ($E_{m,7} = -46$ mV)[18] and indigodisulfonic acid ($E_{m,7} = -125$ mV)[18]. The samples were deaerated by bubbling with Argon freed of O_2 by passage through a column charged with MnO[19] as described by CUSANOVICH AND KAMEN[20]. The previously used Teflon interconnecting tubing between cuvette and vacuum manifold was replaced by 1.4 mm inner diameter, 5.0 mm outer diameter Saran tubing (distributed by Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) to decrease O_2 diffusion into the apparatus.

The redox buffer mixture contained 0.1 M potassium phosphate, 0.5 mM $K_3Fe(CN)_6$, 1.0 mM $FeCl_3$, 10 mM K_2 EDTA, 50 μM phenazine methosulfate (PMS) and a small amount of silicone antifoam solution (Dow Corning Antifoam AF). Stepwise reduction of the reaction system was accomplished by addition of a 2.0 % (w/v) solution of $Na_2S_2O_4$ in 0.1 M potassium phosphate, pH 7.0. To titrate the cytochrome in Fraction A, the particle preparation in the redox buffer mixture in the reference cuvette was kept oxidized. The reduced-*minus*-oxidized difference spectra were recorded for each step of reduction using the 0–0.1, 0.2 Cary slide wire. Absolute spectra were used in titrating the purified cytochrome.

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described previously[8]. For preparative purposes, 24 gels of the cholate-soluble cytochrome complex were run under identical conditions. β-Mercaptoethanol was not included in the protein preincubation mixture because it was found to have no effect on the electrophoresis pattern, but did cause bleaching of the cytochrome visible absorption peaks. The portions of the gels containing colored bands were excised and pooled. Cytochrome was extracted from the crushed gel slices with 0.5 M NaCl, 2 % cholate and 50 mM Tris, pH 8.0. The resultant fractions were desalted on Sephadex G-25 and concentrated by absorption to hydroxylapatite in the absence of detergent. The cytochrome fractions were eluted from hydroxylapatite in concentrated

form by 2 % cholate, 0.2 M NaCl and 0.2 M potassium phosphate, pH 7.0. These fractions were desalted and freeze dried in preparation for future experiments.

RESULTS

Purification

The cytochrome extracted from crude chromatophore starting material was subjected to various purification procedures in an effort to isolate a pure form which could be identified chemically and/or functionally.

The first method tried was DEAE-cellulose chromatography. The desalted protein adsorbed tightly and was eluted at various salt concentrations. None of these fractions showed a significant change in the purity index ($A_{280\,nm}/A_{\gamma}$). Furthermore, a large portion of the cytochrome remained bound to the adsorbent even at high salt concentrations, and could be eluted only if detergent was included in the eluting buffer. DEAE-cellulose chromatography was also unsuccessful when the salt gradient contained 0.1 % Triton X-100 to maintain cytochrome solubility.

Another purification attempt was made by precipitation from organic solvents. Among the solvents tried were: acetone, methanol, dimethyl sulfoxide, dimethyl sulfone, dimethyl formamide, and various combinations of acetone and methanol. In all cases, if enough organic reagent was added to cause precipitation, the cytochrome could not be redissolved without addition of cholate. In some cases the cytochrome could not be redissolved even with detergent. None of the precipitates showed a change in the purity index.

Gel filtration was also ineffective as a purification technique. The cytochrome was eluted in the void volume of a Sephadex G-100 column and as a broad diffuse band from Agarose A-5m with no change in the purity index, with or without detergent included in the eluting buffer. The cytochrome was eluted from Agarose A-5m at a position indicating a molecular size of approximately $2 \cdot 10^5$.

The cytochrome was precipitated by a 20–30 % saturated solution of ammonium sulfate. The redissolved precipitate showed no purification. The amount of cholate present affected the ammonium sulfate concentration at which the cytochrome precipitated. If too much cholate was present (> 0.5 %, w/v), the cytochrome appeared as an insoluble mass floating on the solution. An undetermined amount of cholate precipitated with the cytochrome. If the preparation was repeatedly precipitated with ammonium sulfate, redissolved in Tris buffer, and desalted, the associated cholate was depleted and the precipitation of protein occurred at progressively lower ammonium sulfate concentrations. Ultimately, the cytochrome could be redissolved only by the addition of cholate. This indicated that cholate was essential for solubility.

It was found that the cytochrome was more readily purified if it was extracted from an acetone powder of Fraction A, prepared by the method of THORNBER[9]. Crude chromatophores from cells grown at saturating light intensity are composed chiefly of Fraction A and Fraction B. The isolated Fraction B contains no cytochrome and thus Fraction A is enriched in cytochrome relative to the starting chromatophores.

For the cytochrome preparation, the Thornber recipe was repeated until 6.5 μmoles of Fraction A had been collected (about 0.29 mmoles of total bacteriochlorophyll assuming an absorption constant of 100 mM^{-1} for bacteriochlorophyll at 890 nm). An acetone powder was made and cytochrome extraction performed with 0.5 M NaCl

and 50 mM Tris (pH 8.0) and then with 2 % cholate, 0.5 M NaCl, and 50 mM Tris (pH 8.0) as described previously[8]. Less than 3 % of the total cytochrome present was solubilized by extracting the acetone powder with Tris buffer. Subsequent extraction with detergent solubilized more than 50 % of the remaining cytochrome.

The cholate-solubilized cytochrome was purified on hydroxylapatite by the procedure described in METHODS. Approximately 25 % of the cytochrome in the starting material was recovered as the purified product.

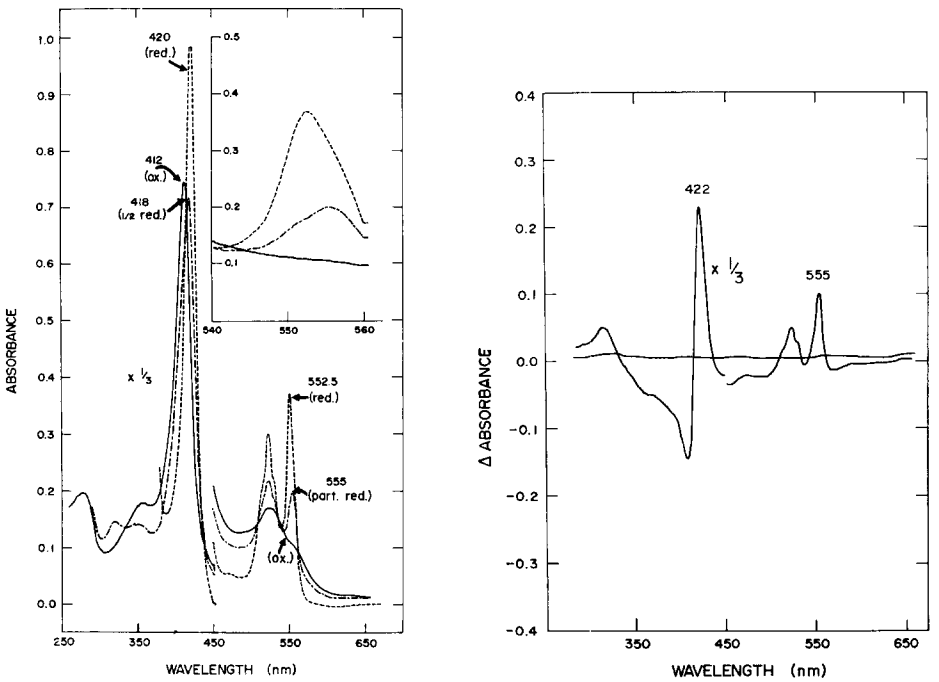

Fig. 1. Absorption spectra of purified *Chromatium* cholate-soluble cytochrome complex. Concentrations in the α peak region, 20 μM in heme; and in the Soret peak region, 6.7 μM. ———, oxidized; — — —, partially reduced with 0.04% β-mercaptoethanol; -------, reduced with $Na_2S_2O_4$.

Fig. 2. Reduced-*minus*-oxidized difference spectrum of *Chromatium* cytochrome *c*-556. The reference sample was completely oxidized, while the sample cuvette contained an equal concentration of cholate-soluble cytochrome partially reduced with 0.04% β-mercaptoethanol. The heme concentrations were 20 μM in the α peak region, and 6.7 μM in the Soret peak region. These concentrations included that of the low potential form of the cytochrome, *c*-552.5, which remained oxidized in both cuvettes.

Characterization

The purified cytochrome exhibited the absorption spectra shown in Fig. 1. Stepwise reduction produced a blue shift in wavelength of the α peak (from 556 nm to 552.5 nm). It was found that 0.05–0.1 % (v/v) mercaptoethanol reduced only the high potential *c*-556 and left the low potential *c*-552.5 cytochrome oxidized. The difference spectrum of *c*-556 is shown in Fig. 2 (*c*-552.5 remained oxidized in both samples) and the difference spectrum of *c*-552.5 is shown in Fig. 3 (*c*-556 was completely reduced in both samples). The difference spectra were virtually identical to those obtained by light-induced absorbance changes in whole cells[1], chromatophores[5] and

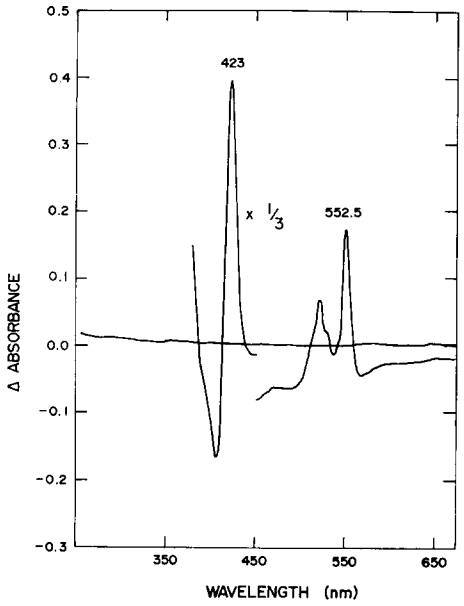

Fig. 3. Reduced-*minus*-oxidized difference spectrum of *Chromatium* cytochrome *c*-552.5. The reference sample was partially reduced with 0.04% β-mercaptoethanol, while the sample cuvette contained cholate-soluble cytochrome reduced with $Na_2S_2O_4$. The heme concentrations were 20 μM in the α peak region, and 6.7 μM in the Soret peak region. These concentrations included that of the high potential cytochrome *c*-556 which was reduced in both cuvettes.

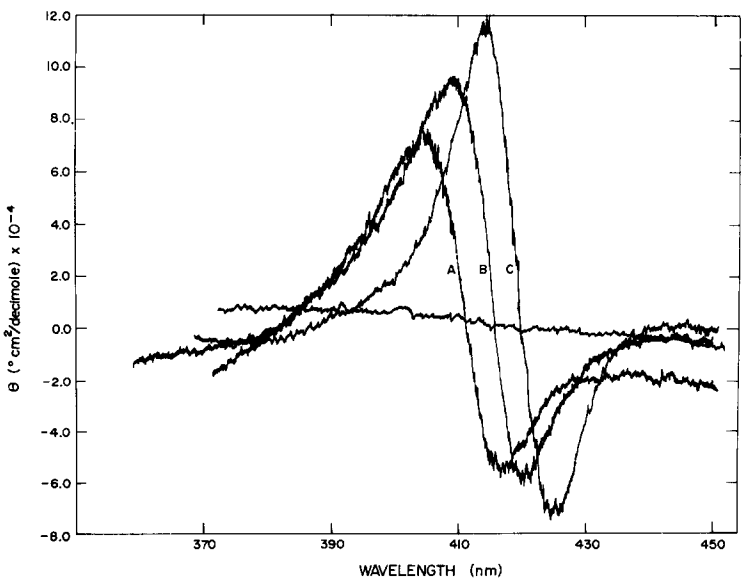

Fig. 4. Circular dichroic spectra of *Chromatium* cholate-soluble cytochrome. Approximate concentration 13 μM in heme. Curve A, oxidized (λ_{max} = 410 nm); Curve B, partially reduced with 0.5% β-mercaptoethanol (λ_{max} = 417 nm); Curve C, reduced with $Na_2S_2O_4$ (λ_{max} = 419.5 nm).

Fraction A[9]. The cytochrome, reduced by $Na_2S_2O_4$ under anaerobic conditions, did not show spectral changes upon bubbling with CO. This was consistent with the lack of CO-binding cytochrome in purified chromatophores[19].

A low temperature (77°K) spectrum of the reduced α region showed a complex peak. The second derivative spectrum of this region pinpointed the major peak at 549 nm with a large shoulder at 555 nm and a lesser shoulder at 547 nm.

Fig. 4 shows the circular dichroism spectra of the cytochrome in the Soret region. The sigmoid shape of the curve was characteristic of all three oxidation states of the complex cytochrome. The circular dichroism curve always crossed the baseline at the exact maximum absorbance of the Soret peak. No change in this spectrum was noted on freezing and thawing.

Sodium dodecyl sulfate acrylamide gel electrophoresis of the purified cytochrome showed multiple bands (Fig. 5). This was in contrast to the single cytochrome band found when Fraction A was run on the gels. These bands were excised, extracted from the gels of several runs, concentrated and rerun. Each isolated fraction then migrated as a single component in its original position. Since sodium dodecyl sulfate and urea denatured the cytochromes, no spectral identification of *c*-556 or *c*-552.5 could be made. Amino acid analyses of each band showed identical compositions, so the multiple banding was thought to be an artifact possibly produced by differing amounts of cholate associated with the protein. Extraction of the cytochrome with glacial acetic acid and other organic solvents for cholate prior to electrophoresis failed to abolish the multiple banding. The observed approximate molecular weights for the major components were 45000, 29000 and 23000. No colorless proteins were

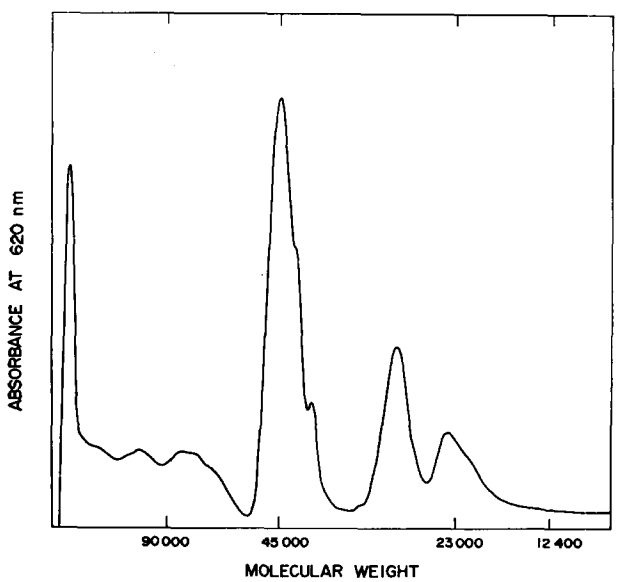

Fig. 5. Sodium dodecyl sulfate acrylamide gel electrophoresis of purified *Chromatium* cholate-soluble cytochrome. The sharp peak at the high molecular weight end of the scan is due to the refractive index change as the gel enters the measuring beam. The gel was stained with Amido black, and all stained bands correspond to colored bands before staining. The gels were run for 4 h at 15 mA/gel.

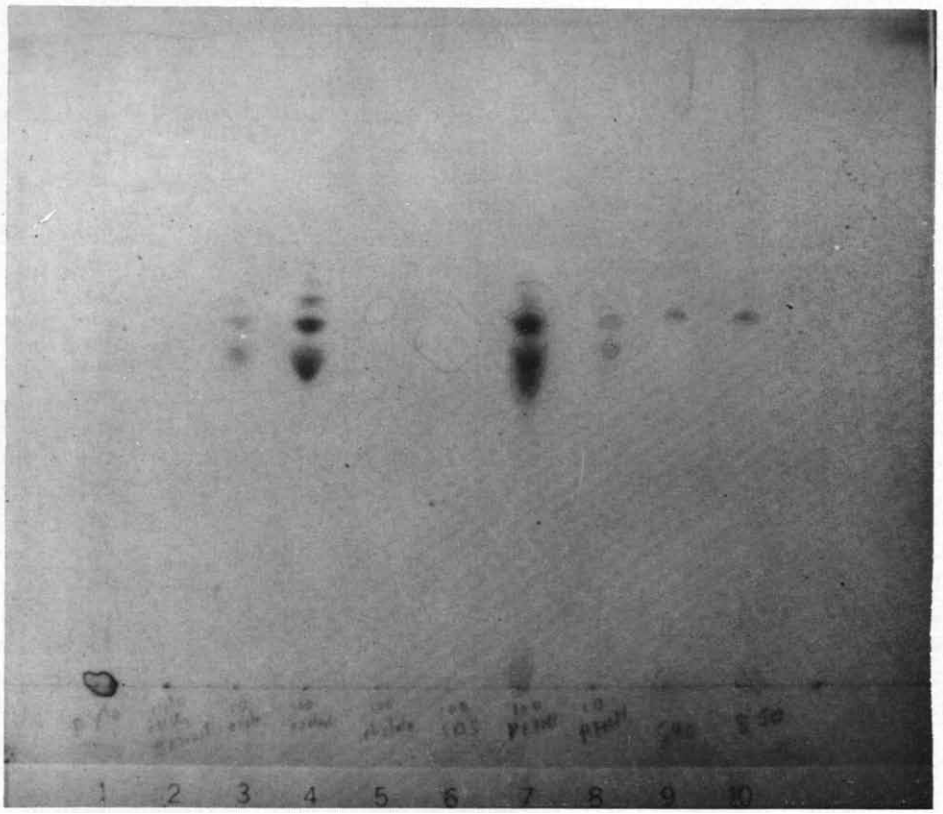

Fig. 6. Thin layer chromatography of phospholipids and membrane fractions. Samples were applied to a 0.25-mm thick pre-coated Silica gel plate (E. Merck) which was developed for 4 h in diisobutyl ketone–acetic acid–water–ethanol (80:50:10:10, v/v/v/v). The plate was stained with I_2 vapor and then with phospholipid spray[16]. The samples are: (1) 1 mg cholate-soluble cytochrome; (2) chloroform extract from 0.5 mg cholate-soluble cytochrome; (3) 10 μg Azolectin (Associated Concentrates, Inc.); (4) 100 μg Azolectin; (5) 100 μg potassium cholate; (6) 100 μg sodium dodecyl sulfate; (7) 100 μg phosphatidyl ethanolamine; (8) 10 μg phosphatidyl ethanolamine; (9) 50 μg Fraction A; and (10) 50 μg Fraction B.

detected in the purified preparation. If an impure cytochrome preparation was processed, the same cytochrome bands appeared, but in addition, contaminating protein bands could be seen.

The cytochrome was applied to a thin layer chromatography plate with various lipid and detergent standards to see if the multiple bands could be due to lipid–cytochrome aggregation. The results and details of the method are presented in Fig. 6. Although phospholipid appeared in several spots and could be seen to be present in both Fractions A and B, no phospholipid was detected in the cytochrome preparation. Sodium dodecyl sulfate was present in both Fractions A and B but not in the cytochrome, while the reverse was true of cholate.

The amino acid composition of the protein is given in Table I. The protein was very slow to hydrolyze and some residues (notably valine) increased in each 24-h increment. The analysis differed from all of the known buffer-soluble proteins. No mixture of amino acid analyses of the soluble *Chromatium* proteins, *viz.* high potential

TABLE I

AMINO ACID ANALYSIS OF PURIFIED CHOLATE-SOLUBLE CYTOCHROME

Amino acid	*Residues/heme*		
	24 h hydrolysis	*48 h hydrolysis*	*72 h hydrolysis*
Asp	7.56	8.10	7.96
$MetSO_2$	—	—	—
Thr	4.60	5.21	5.50
Ser	4.70	4.84	4.45
Glu	9.53	10.06	10.60
Pro	7.69	7.89	8.95
Gly	5.09	5.32	5.50
Ala	10.68	10.95	11.35
Cys	N.D.*	N.D.	N.D.
Val	4.68	6.38	7.32
Met	1.42	1.42	1.53
Ile	1.14	2.05	2.35
Leu	4.46	5.01	5.00
Tyr	3.23	3.70	3.74
Phe	1.45	1.60	1.55
His	1.09	1.29	1.41
Lys	2.77	3.07	3.13
Trp	N.D.	N.D.	N.D.
Arg	2.87	3.02	3.26

* N.D., not determined.

TABLE II

AMINO AND CARBOXYL END GROUP ANALYSES OF THE CHOLATE-SOLUBLE CYTOCHROME COMPLEX

Amino acid	*Amino-terminal*		*Carboxyl-terminal*	
	Residues per heme	*Residues per 45000 daltons*	*Residues per heme*	*Residues per 45000 daltons*
Asp	0.019	0.095	0.028	0.14
Ser			0.073	0.36
Glu	0.036	0.18	0.021	0.10
Pro	0.023	0.115		
Gly	0.025	0.125	0.044	0.22
Ala	0.039	0.195	0.046	0.23
Val	0.018	0.090		
Leu			0.029	0.15

cytochrome *c*-553(550)[7], low potential cytochrome *c*-553 or cytochrome *c'*[21], produced a composition high enough in proline or alanine, yet low enough in glycine and glutamic acid to correspond to that obtained.

The amino-terminal analyses (Table II) were performed by Edman degradation followed by phenylthiohydantoin-derivative hydrolysis and amino acid analysis. This method yielded about 60% the theoretical values in control experiments on other proteins. Table II shows that recoveries in the experiments on the cholate-soluble cytochrome complex were very poor with a display of considerable heterogeneity. Several amino acids which were expected to disappear on hydrolysis were

absent in thin layer chromatography except for glutamine[22]. The unusually low yields may have resulted because the cytochrome failed to dissolve well in the coupling buffer. This, however, should not have affected the overall yield (Dr. R. Doolittle, personal communication).

The carboxyl-terminal analyses also showed heterogeneity and low yields (Table II). The carboxyl-terminal amino acid may have been basic and so not recovered in good yield by the hydrazinolysis procedure performed.

The redox potential of the extracted cytochrome was measured as described in METHODS. A plot of the change of α peak absorbance against redox potential is shown in Fig. 7. There were two major redox components, with midpoint potentials of +325 mV for the high potential cytochrome (*c*-556) and about +8 mV for the low potential cytochrome (*c*-552.5). Both cytochromes yielded a normal linear Nernst plot of $E_{h,7}$ *versus* log (ox/red) with n approximately equal to one (*i.e.* approx. 60 mV/log). The midpoint potentials were independent of the redox mediators used, the pH range between 6 and 8, and the ATP concentration up to 1 mM. The ratio of the maximum absorbance changes in the α band for high and low potential cytochromes as determined by the relative heights between plateau regions of Fig. 7 was about 1–3. This may not have been the mole ratio, however, because of uncertainty about the equivalence of the extinction coefficients of the two cytochrome components. The high potential cytochrome component had an asymmetrical α peak and thus, possibly, a relatively small extinction coefficient as found to be the case with cytochrome *c*-553(550) from *Chromatium*[7]. To meet this constraint, the best integral mole ratio of *c*-556:*c*-552.5 was 1:2. The ratio of the cytochromes ($A_{556\text{ nm}}/A_{552,5\text{ nm}}$) remained the same throughout the purification procedure. If the cytochrome complex was

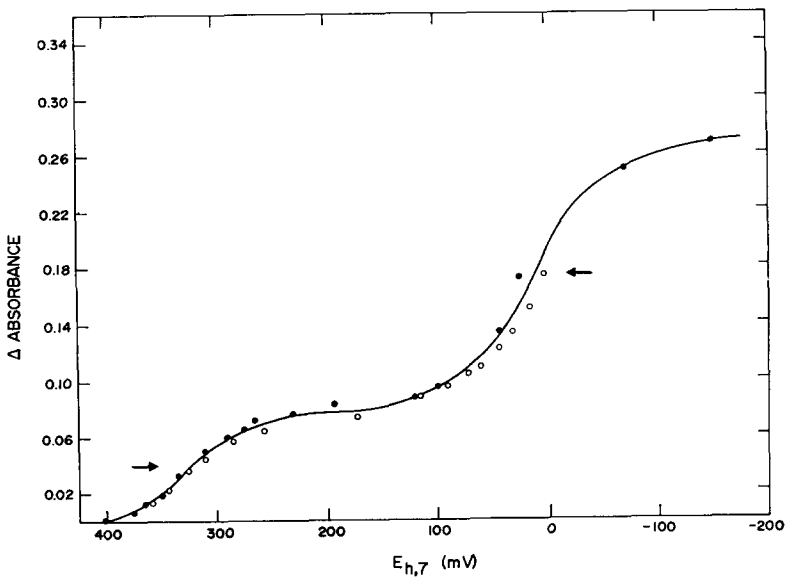

Fig. 7. Redox titration of purified *Chromatium* cholate-soluble cytochrome. ●, reductive titration with 2% (w/v) $Na_2S_2O_4$; ○, oxidative titration with 100 mM $K_3Fe(CN)_6$. Arrows indicate the approximate midpoint potentials for cytochrome *c*-556 (+325 mV) and cytochrome *c*-552.5 (+8 mV).

incubated in excess cholate for prolonged periods of time, the high potential cytochrome failed to yield a definite sigmoid titration curve, possibly indicating *c*-556 lability to prolonged exposure to cholate.

A chemical redox titration of the cytochrome in Fraction A is shown in Fig. 8. The midpoint potentials for *c*-556 (+345 mV) and for *c*-552.5 (−8 mV) were nearly the same as those for the cholate-extracted cytochrome. Although there was a small background decrease in absorbance in the α region with decreasing redox potential, the ratios of the α region changes were similar to those of the extracted cytochrome. Thus the cytochrome complex appeared to retain its native biological properties even after extraction by 2 % cholate.

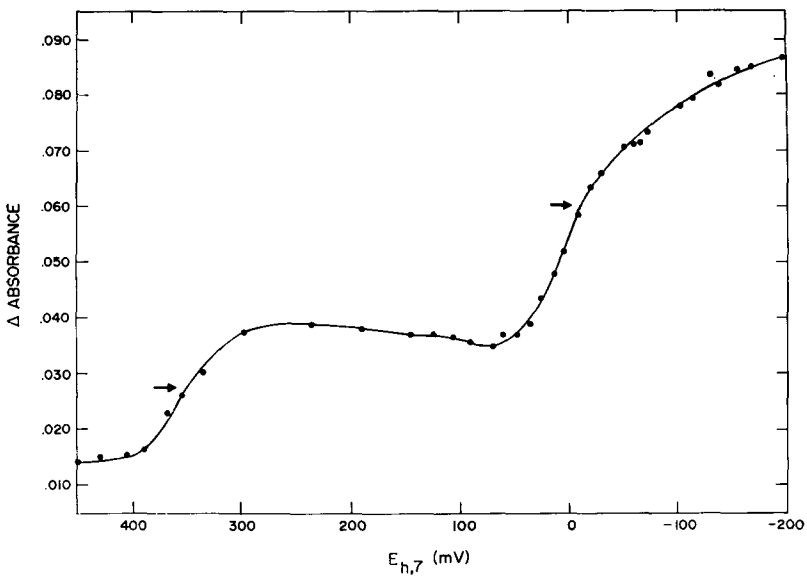

Fig. 8. Redox titration of the cytochromes in Fraction A. All data were obtained by reductive titration with 2% (w/v) $Na_2S_2O_4$. The approximate midpoint potentials indicated by the arrows are: *c*-556, +345 mV; and *c*-552.5, −8 mV.

DISCUSSION

The cholate-soluble cytochrome complex

The major portion of the total *Chromatium* cytochrome remains membrane-bound after cell breakage and fractionation[8]. A small amount of iron-containing protein which can be extracted in buffer from the acetone-extracted chromatophores consists of previously demonstrated buffer-soluble iron-containing proteins. This buffer-soluble fraction, containing flavocytochrome *c*-553, cytochrome *c'* and high-potential iron–sulfur protein (HiPISP, known also as HIPIP), is not present in the purified Fraction A which still performs all of the light-induced cytochrome oxidations observed in the chromatophore preparation. Therefore the buffer-soluble cytochrome may represent proteins in non-specific association with the membrane.

The cytochrome which remains attached to the membrane is present in large amounts in all growth conditions tested and can be quantitatively solubilized with

cholate. The purified cytochrome complex contains two components, designated "cytochrome *c*-556", with a high redox potential, $E_{m,7} \simeq 340$ mV, and "cytochrome *c*-552.5", with a low redox potential, $E_{m,7} \simeq 0$ mV. The two components have resisted all efforts to separate them.

The cholate-soluble cytochrome complex is different from the buffer-soluble cytochromes in several respects. (1) Chromatographic characteristics using DEAE-cellulose, hydroxylapatite, and Sephadex G-100 are all different from those exhibited by any of the soluble cytochromes (flavocytochrome *c*-553, cytochrome *c'*, and cytochrome *c*-553(550)) under similar conditions. (2) Sodium dodecyl sulfate acrylamide gel electrophoresis of flavocytochrome *c*-553 with or without added cytochrome *c*-553(550) does not produce the pattern observed with cholate-soluble cytochrome (Fig. 5). (3) The absorption spectra of the cholate cytochrome complex cannot be identified with that of any combination of soluble cytochromes. The reduced-*minus*-oxidized difference spectrum of the high potential cytochrome *c*-556 component of the cholate-extracted cytochrome complex (Fig. 2) differs from that of the soluble high potential cytochrome *c*-553(550)[7] in the positions of the α and Soret peaks. Furthermore, the ratios of Soret/α absorption peaks for these two cytochromes are different. (4) Comparison of amino acid compositions shown in Table I, and the amino acid compositions of flavocytochrome *c*-553[21], cytochrome *c'*[21] and of cytochrome *c*-553(550)[7] precludes identification of the cholate-soluble cytochrome complex with any one or combination of soluble cytochromes, as remarked previously.

Unfortunately the amino acid end group analyses of the detergent-solubilized cytochrome complex are inconclusive for identifying the cytochromes, owing to failure to obtain reliable values for the cholate-soluble cytochrome. Amino- and carboxyl-terminal determinations indicate heterogeneity (Table II), but these are unreliable owing to the poor yields obtained. The cholate-soluble cytochrome was found to contain no colorless bands on sodium dodecyl sulfate acrylamide gel electrophoresis; it contained no phospholipid but did retain a trace of cholate (approximately 50 μg/mg protein as estimated by thin layer chromatography).

The components of the purified cholate-soluble cytochrome complex are identified with the two cytochrome species which undergo light-induced oxidation in both cells and chromatophores on the following bases:

(a) The oxidized-*minus*-reduced difference spectrum of purified cytochrome *c*-556 is identical with the light-induced spectrum of the cytochrome which is oxidized at high redox potential ($E_{h,7} > 250$ mV). Similar identity is shown between the difference spectrum of purified cytochrome *c*-552.5 and the spectrum of the cytochrome undergoing light-induced oxidation of low redox potentials. The minor variations in the α peak position of cytochrome *c*-556 (from 555 nm to 557 nm) in the purified form as well as in light-induced spectra are not as yet understood.

(b) CUSANOVICH *et al.*[5] showed that chromatophore-bound cytochromes do not bind CO whereas the soluble cytochromes *c*-553 and *c'* do bind CO. They explain this discrepancy by suggesting that CO does not penetrate the membrane material. The fact that cholate-soluble cytochrome does not bind CO suggests that it, and not flavocytochrome *c*-553 or cytochrome *c'*, is the major cytochrome component in chromatophores.

(c) The position of the major component of the α peak absorption in the liquid nitrogen temperature spectrum is 549 nm. This is consistent with the 550 nm α peak

minimum observed by KIHARA AND DUTTON[23] in light-induced cytochrome oxidation at these temperatures.

(d) The redox potentials of the purified cytochrome *c*-556 and cytochrome *c*-552.5 are +325 mV and +8 mV, respectively (Fig. 7). The potentials of these cytochromes chemically titrated while still membrane bound are +345 mV and −8 mV (Fig. 8). These values are consistent with the redox potentials estimated from light-induced absorbance changes[5].

Circular dichroism spectra of the cholate-soluble cytochrome complex in various redox states show sigmoid curves for the γ peaks (Fig. 4). Such results have been interpreted[24] as indications of heme–heme interaction. If this hypothesis is valid, all of the hemes in the cytochrome complex may be interacting, because the position of the sigmoid circular dichroism curve shifts with the position of the Soret band at three different cytochrome redox states: cytochromes *c*-556 and *c*-552.5 both oxidized; cytochrome *c*-556 reduced and cytochrome *c*-552.5 oxidized; cytochromes *c*-556 and *c*-552.5 both reduced. If either the low or the high potential heme did not participate in the heme–heme interaction, one would expect asymmetry of the circular dichroic curve for one or more of the redox states.

Another indication of the polyheme nature of this cytochrome complex comes from the molecular weight determination. The amino acid composition (Table I) gives a maximum of 90 amino acids per heme, while sodium dodecyl sulfate acrylamide gel electrophoresis (Fig. 5) indicates a molecular weight of about 45 000 for the predominant species. This is consistent with 4 or 5 hemes per cytochrome complex (*e.g.* 360–450 amino acids per unit).

These considerations all bear on the location of cytochrome *c*-552.5 and cytochrome *c*-556 in the photosystem. Sodium dodecyl sulfate acrylamide gel electrophoresis and circular dichroic spectral properties of the cholate-soluble cytochrome complex indicate a close association between the two cytochromes, although the physiological significance of detergent-treated samples is unclear. The possible existence of cytochromes *c*-556 and *c*-552.5 in the same cytochrome complex has some interesting implications, *e.g.* (1) the cytochromes probably function in the same photosystem; (2) their interaction in all cell reactions would be facilitated owing to the close association of their hemes, and the process of energy conservation by a photophosphorylation site between cytochrome *c*-552.5 and cytochrome *c*-556 could be accomplished by a single protein conformational change. The available data do not warrant further speculation.

The multiple bands of the purified cholate-soluble cytochrome in sodium dodecyl sulfate acrylamide gel electrophoresis may arise owing to a number of factors. (1) The 45 000 molecular weight band may be an aggregated form of those giving the smaller bands. (2) Residual bound cholate may cause aberrant electrophoretic behavior. (3) *Chromatium* protease action may initiate degradation of the cytochromes once they are released from the membrane; if this is the case, part of the cytochrome peptide chain would be lost and so affect the amino acid composition. Because the major cytochrome band coincides with the sole cytochrome band found in sodium dodecyl sulfate acrylamide gel electrophoresis of particle preparation, it is concluded that the primary band is at least closely related to the functional cytochrome complex of the photoreactive membrane and that this complex is composed of two different cytochromes, namely cytochrome *c*-552.5 and cytochrome *c*-556.

Finally, failure to separate and isolate unique proteins containing exclusively the cytochrome *c*-556 or cytochrome *c*-552.5 hemes has prevented determination of extinction coefficients for these cytochromes. Although the total heme can easily be quantitated, the relative contributions of cytochrome *c*-556 and cytochrome *c*-552.5 are not known. Assuming a ratio of 2 cytochromes *c*-552.5 per cytochrome *c*-556, as discussed in RESULTS, the following reduced-*minus*-oxidized extinction coefficients are calculated from Figs. 2 and 3: high potential cytochrome, ε_{red-ox} (556 nm) = 15.1 mM^{-1} and ε_{red-ox} (422 nm) = 103 mM^{-1}; low potential cytochrome, ε_{red-ox} (552.5 nm) = 13.1 mM^{-1} and ε_{red-ox} (423.5 nm) = 89 mM^{-1}.

ACKNOWLEDGMENTS

Instruction by Dr. J. P. Thornber in preparation of Fraction A is gratefully acknowledged. Financial support for these researches was furnished by grants-in-aid from the National Institutes of Health (HD-01262) and National Science Foundation (GB-7033X). S. K. was supported by a National Institutes of Health training grant (U.S. Public Health Service 2-T01-01045-08).

REFERENCES

1 J. M. OLSON AND B. CHANCE, *Arch. Biochem. Biophys.*, 88 (1960) 26.
2 J. M. OLSON AND B. CHANCE, *Arch. Biochem. Biophys.*, 88 (1960) 40.
3 D. DEVAULT AND B. CHANCE, *Biophys. J.*, 6 (1966) 825.
4 S. MORITA, M. EDWARDS AND J. GIBSON, *Biochim. Biophys. Acta*, 109 (1965) 45.
5 M. A. CUSANOVICH, R. G. BARTSCH AND M. D. KAMEN, *Biochim. Biophys. Acta*, 153 (1968) 397.
6 R. G. BARTSCH, in H. GEST, A. SAN PIETRO AND L. P. VERNON, *Bacterial Photosynthesis*, Antioch Press, Yellow Springs, Ohio, 1963, p. 475.
7 M. A. CUSANOVICH AND R. G. BARTSCH, *Biochim. Biophys. Acta*, 189 (1969) 245.
8 S. J. KENNEL, R. G. BARTSCH AND M. D. KAMEN, *Biochim. Biophys. Acta*, 234 (1971) 458.
9 J. P. THORNBER, *Biochemistry*, 9 (1970) 2688.
10 H. W. SIEGELMAN, G. A. WIECZOREK AND B. G. TURNER, *Anal. Biochem.*, 13 (1965) 420.
11 P. EDMAN, *Ann. N.Y. Acad. Sci.*, 88 (1960) 602.
12 R. F. DOOLITTLE, *Biochem. J.*, 94 (1965) 742.
13 K. DUS, S. LINDROTH, R. PABST AND R. M. SMITH, *Anal. Biochem.*, 14 (1966) 41.
14 K. DUS, S. LINDROTH, R. PABST AND R. M. SMITH, *Anal. Biochem.*, 18 (1967) 532.
15 K. DUS, R. G. BARTSCH AND M. D. KAMEN, *J. Biol. Chem.*, 237 (1962) 3083.
16 W. STOECKENIUS AND W. H. KUNAU, *J. Cell Biol.*, 38 (1968) 337.
17 W. BUTLER, in A. SAN PIETRO, *Methods in Enzymology*, Vol. 23, Academic Press, New York, 1971, in the press.
18 W. M. CLARK, *Oxidation-Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore, Md., 1960.
19 P. A. HERSCH, *Anal. Chem.*, 32 (1960) 1030.
20 M. A. CUSANOVICH AND M. D. KAMEN, *Biochim. Biophys. Acta*, 153 (1968) 376.
21 R. G. BARTSCH, M. L. COVAL AND M. D. KAMEN, *Biochim. Biophys. Acta*, 51 (1961) 241.
22 P. EDMAN AND J. SJÖQUIST, *Acta Chem. Scand.*, 10 (1956) 1507.
23 T. KIHARA AND P. L. DUTTON, *Biochim. Biophys. Acta*, 205 (1970) 196.
24 R. G. BARTSCH, T. E. MEYER AND A. B. ROBINSON, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZO, *Structure and Function of Cytochromes*, University Park Press, Baltimore, Md., 1968, p. 443.