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Purification and Properties of Cytochrome b_1 from *Escherichia coli*

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Cytochrome b_1 occurs widely in microbes (*1*) and is the predominant cytochrome component in the *coli-aerogenes* group of bacteria. Its function as an electron carrier in the terminal oxidation systems has been established in various organisms with succinate (*2—4*), NADH (*5*), formate (*6—8*) and pyruvate (*9, 10*) as substrates. It has further been shown that in *Escherichia coli* this cythchrome is involved in the electron-transport chain responsible for nitrate respiration (*11—16*). The nature of this important hemoprotein has, however, not yet been well characterized, since at least in *E. coli* it is localized in the particulate cell fraction (*12, 16*) and, until recently, had resisted attempts at its solubilization.

In a recent paper, Wrigley and Linnane (*6*) reported the solubilization of a complex consisting of cytochrome b_1 and formate dehydrogenase from the particulate fraction of *E. coli*. More recently, Deeb and Hager (*10*) have also obtained a soluble preparation of the cytochrome from the same source. In independent work, we have also succeeded in solubilizing a preparation, also from *E. coli*, containing formate denhydrogenase and nitrate reductase in addition to cytochrome b_1 (*7, 8*). Although some properties of cytochrome b_1 in the last-mentioned preparation have been described (*8*), it seemed desirable to purify it further to characterize it physicochemically in more detail.

This communication describes the purification, to more satisfactory extents, of cytochrome b_1 from the particulate fraction of *E. coli* and reports some properties of the purified preparation.

MATERIALS AND METHODS

Bactererial Cells—*E. coli*, Yamagutchi strain, was aerobically cultivated with vigorous shaking at 37°C for 16 hours in a medium containing, in g. per liter, the following: glucose, 10; polypepton (Wako), 5; meat exract (Kyokuto), 5; KNO_3, 2.5; sodium formate, 2.5; K_2HPO_4, 1; Casamino acids (Difco), 0.5; yeast-extract powder (Daigo), 0.5; pH 7.2 to 7.4 adjusted with NaOH. The cells were harvested in a Sharples centrifuge, washed once with cold water, and frozen at −20°C until use. In several preparations the cells grown anaerobically as described previously (*8*) were used, but no essential difference was noticed among the products purified from the two types of cells.

Particulate Preparation — The frozen cells were ground with alumina and exracted as described by Taniguchi and Itagaki (*14*). The extract was centrifuged at $2,000 \times g$ for 20 minutes and the particles in the supernatant were then sedimentated by centrifugation at $78,000 \times g$ for 30 minutes. When the particulate fraction thus obtained was very viscous, it was resuspended in 5 mM Tris-HCl buffer, pH 7.1, containing 1 mM $MgCl_2$, 1 mg. per liter of crystalline pancreatic deoxyribonuclease [EC 3. 1. 4. 5.] and 2.5 mg. per liter of crystalline pancreatic ribonuclease [EC 2. 7. 7. 16] and the mixture was dialyzed in a nitrogen atmosphere at 0°C for about 15 hours against 5 mM Tris-HCl buffer, pH 7.1, containing 1 mM $MgCl_2$. The particulate fraction, now freed from viscous materials, was recovered by centrifugation at $78,000 \times g$ for 30 minutes.

Analytical Procedures—Cytochrome b_1 and protoheme were determined as in the previous report (*8*). Flavin was assayed by the lumiflavin method (*17*) using an Aminco recording spectrofluorometer; the samples were excited by the light at 365 mμ and the fluorescence intensity was read at 520 mμ. For the analyses of iron, deoxycholate, RNA, DNA and protein were used the methods of Sandell (*18*), Mosbach *et al.* (*19*), Kerr and Seraidarian (*20*), Dische and Schwarz (*21*) and Lowry *et al.* (*22*) respectively. The activities of formate, succinate and glucose dehydrogenases [EC 1. 2. 2. 1, 1. 3. 99. 1, 1. 1. 1. 47] were assayed by measuring the decoloriza-

tion of methylene blue. Formate dehydrogenase was also determined by the nitrate reductase method as described previously (*8*). The oxidation of NADH and NADPH was followed spectrophotometrically at 340 mμ. The nitrate reductase [EC 1. 9. 6. 1] activity was measured by using reduced methyl viologen as electron donor as described by Taniguchi and Itagaki (*14*). Electrophoresis was carried out in a Hitachi model HTB Tiselius-type apparatus. A Hitachi model UCA-1 analytical ultracentrifuge was used for the sedimentation analysis. All the spectrophotometric measurements were carried out in a Cary model 14 spectrophotometer.

Determination of Oxidation-Reduction Potential—The oxidation-reduction potential of cytochrome b_1 was measured at 25°C in the ferric-ferrous oxalate system (*23*). In a spectrophotometer cuvette equipped with a tonometer-type glass chamber (*24*) were placed 0.5 ml. of 0.5 *M* potassium oxalate, 1.0 ml. of 0.1 *M* phosphate buffer, pH 7.0, 0.2 ml. of 0.01 *M* $FeCl_3$, and 0.5 ml. of cytochrome b_1 dissolved in 0.1 *M* phosphate buffer, pH 7.0. After replacing the atmosphere by pure nitrogen by repeated evacuation and flashing, the content was titrated with 0.01 *M* $FeSO_4$ with the aid of a Hamilton microliter syringe (0.1 ml. full scale) inserted into the rubber stopper. After each addition of $FeSO_4$, the content was gently shaken for 5 to 10 minutes and the spectrum was measured. The reduction of the cytochrome was determined from the intensity of α-band at 559 mμ and the normal oxidation-reduction potential was obtainted graphically by the method of Hill (*23*).

Splitting and Reconstruction—To 1 volume of cytochrome b_1 dissolved in 0.05 *M* phosphate buffer, pH 7.0, were added 4 volumes of acid acetone (1 vol. of conc. hydrochloric acid in 500 vol. of acetone) (*25*) which had been chilled to $-50°$ to $-60°$C. The mixture was homogenized with a glass rod and the apoprotein moiety was separated from the extracted heme by centrifugation at 29,000$\times g$ for 5 minutes. The apoprotein moiety was once more treated with acid acetone and the apoprotein precipitate recovered by centrifugation was homogenized with an appropriate amount of 0.2 *M* Tris-HCl buffer, pH 8.5. The apoprotein suspension thus obtained contained 3 mg. of protein per ml. and showed no absorption bands in the visible region even after the addition of dithionite. Crystalline hemin chloride prepared from beef blood, on the other hand, was dissolved in a minimum amount of 0.01 *N* NaOH and diluted with 0.1 *M* phosphate buffer, pH 8.0, so as to make the final concentration 0.66 m*M*. To 2 ml. of the apoprotein suspension was added 0.1 ml. of the hemin solution and the reconstruction of cytochrome b_1 was measured spectrophotometrically.

Chemicals—Hydroxylapatite was prepared according to Main *et al.* (*26*). Ubiquinone with a side chain consisting of eight isoprenoid units (UQ_8) was extracted and purified from *E. coli* Yamagutchi strain by a modification of the method of Lester and Crane (*27*). Reduced UQ_8 was prepared as described elsewhere. Ethyl isocyanide was synthesized from ethyl iodide and silver cyanide (*28*). Cytochrome *c* was isolated and crystallized from beef heart as described by Hagihara *et al.* (*29*). Reduced vitamin K_3 was prepared as described by Fieser (*30*).

RESULTS

Purification of Cytochrome b_1

Solubilization—Cytochrome b_1 was solubilized from the particulate fraction of *E. coli* essentially as described previously (*7, 8*). The particulate fraction was suspended at a final concentration of 12 to 18 mg. of protein per ml. in a medium containing 0.1 % sodium deoxycholate, 0.05 % freeze-dried crude venom of the snake *Trimeresurus flavoviridis*, 10 μM EDTA and 0.05 *M* Tris-HCl buffer, pH 8.0. The mixture was incubated at 0°C for 16 to 18 hours in a nitrogen atmosphere, and then centrifuged at 78,000$\times g$ for 30 minutes. The reddish brown supernatant thus obtained contained 40 to 60 per cent of the cytochrome b_1 originally present in the particulate fraction. The recovery of the cytochrome was sometimes lower than 40 per cent, especially when older preparations of the venom were used. It was possible to improve the recovery in such cases by subjecting the particulate fraction to sonic oscillation (9 kc.) for 20 minutes, by doubling the concentrations of deoxycholate and the venom, and by raising the pH of the incubation mixture to 8.5.

First Ammonium Sulfate Fractionation—This and all the subsequent manipulations were carried out at 0° to 4°C. Finely powdered ammonium sulfate was added to 70 % saturation to the solubilized supernatant with vigorous stirring and the mixture was allowed to stand for 30 minutes. The reddish brown precipitate was collected by centrifugation at 10,000$\times g$ for 20 minutes and suspend in 0.1 *M*

Tris-HCl buffer, pH 7.1, corresponding to one tenth the volume of the original supernatant. The suspension was dialysed in a nitrogen atmosphere for 16 to 18 hours against a large volume of 0.01 M Tris-HCl buffer, pH 7.1. The dialysed solution was centrifuged at 10,000 $\times g$ for 20 minutes and the precipitate was discarded.

Second Ammonium Sulfate Fraction at pH 5.3—The pH of the supernatant was adjusted to 5.3 by adding carefully M acetate buffer, pH 5.1. This treatment resulted in the precipitation of brown proteins. To the solution containing the precipitated proteins was then added powdered ammonium sulfate to 30 % saturation and the mixture was allowed to stand for 30 minutes. The mixture was centrifuged at 10,000 $\times g$ for 20 minutes and the precipitate was dissolved in 5 mM Tris-HCl buffer, pH 7.1, corresponding to 1/30 to 1/40 of the volume of the original solubilized supernatant. The solution was dialysed overnight in a nitrogen atmosphere against the same buffer and the insoluble material was removed by centrifugation.

Third Ammonium Sulfate Fractionation at pH 8.0—The solution obtained in the preceding step was adjusted to pH 8.0 by the addition of 0.5 M Tris-HCl buffer, pH 8.0. The solution was then made 45 per cent saturated with respect to ammonium sulfate with saturated solution which had been adjusted to pH 8.0 with ammonia. After standing for 20 to 30 minutes, the mixture was centrifuged at 10,000 $\times g$ for 30 minutes and the pellet was discarded. To the supernatant was further added the ammonium sulfate solution till 60 % saturation and the mixture was allowed to stand for 20 minutes. The resulting reddish precipitate was collected by centrifugation at 78,000 $\times g$ for 30 minutes, dissolved in a small amount of 0.05 M Tris-HCl buffer, pH 7.1, and dialysed anaerobically for 16 to 18 hours against 5 mM Tris-HCl buffer, pH 7.1. The insoluble material, if any, was removed by centrifugation at 29,000 $\times g$ for 15 minutes.

Hydroxylapatite Column Chromatography—The dialysed solution obtained in the third ammonium sufate fractionation (15 to 20 ml.) was applied to a hydroxylapatite column (2 $\times$ 20 cm. or 3 $\times$ 15 cm.) previously buffered with 0.01 M phosphate buffer, pH 7.0, and the column was washed with 5 mM phosphate buffer, pH 7.0. When the washing solvent was changed to 0.1 M phosphate buffer, pH 7.0, a yellowish band began to migrate along the column and was eluted out. As judged from its adsorption spectrum, this yellowish substance appeared to be a denaturation product of cytochrome b_1. After the yellow band had been eluted out, the elution was continued with 0.25 M pyrophosphate buffer, pH 7.0, as solvent. A reddish brown band thereby migrated along the column and was eluted out. This fraction containing purified cytochrome b_1 was dialysed anaerobically for 16 18 hours against 0.05 M phosphate buffer, pH 7.0.

TABLE I

Summary of Purification of Cytochrome b_1

Purification step	Volume	Protein	Cytochrome b_1			
			Total content[1]	Specific content[1]	E_{280}/E_{415}	Yield
	(ml.)	(mg.)	(mμmole)	(mμmole / mg. protein)		(%)
Solubilized supernatant	2,300	15,600	4,460	0.29	—	100
1 st $(NH_4)_2SO_4$ ppt. (0–70%)	425	7,580	2,860	0.38	5.60	64
2 nd $(NH_4)_2SO_4$ ppt. (0–30%), pH 5.3	150	2,380	1,880	0.79	4.50	42
3 rd $(NH_4)_2SO_4$ ppt. (45–60%), pH 8.0	17	226	672	2.97	2.98	15
Hydroxylapatite column eluate	17	140	446	3.20	2.72	10

1) Based on the assumption that 1 mole of heme is contained per mole of cytochrome.

Summary of Purification Experiments — In Table I are summarized the results of a typical purification experiment in which 850 g. (wet weight) of frozen cells were used as the starting material. Although the results varied from experiment to experiment, the recovery of cytochrome b_1 in the final products was usually 10 to 15 per cent from the solubilized supernatant or 4 to 8 per cent from the particulate fraction. The final preparations contained 3.0 to 4.6 mμmoles of protoheme per mg. of protein, corresponding to an 11- to 15-fold purification over the solubilized supernatant. As another measure of the purity, the ratio of absorption at 280 mμ (due to protein) to that at 415 mμ (Soret peak of the oxidized cytochrome) was measured. The E_{280}/E_{415} ratio of the purified preparations ranged from 1.95 to 3.0.

Properties of Purified Cytochrome b_1

Electrophoretic Behavior—When a purified preparation with an E_{280}/E_{415} ratio of 2.98 was analysed in a Tiselius-type electrophoresis apparatus, it behaved as an almost homogeneous protein as shown in Fig. 1, though the presence of small amounts of impurities could definitely be detected. Experiments conducted at pH 6.5, 7.0 and 8.0 gave similar electrophoretic patterns, though the mobility increased with increasing alkalinity. The main colored peak migrated to the anode even at pH 6.5, indicating that the cytochrome is an acidic protein.

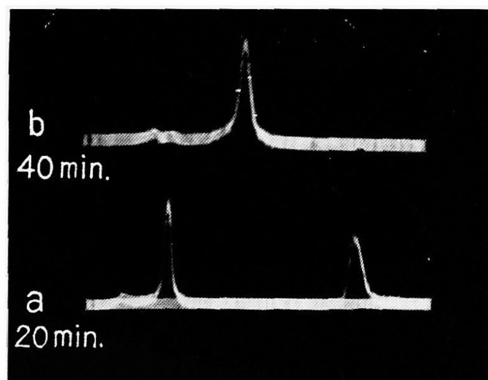
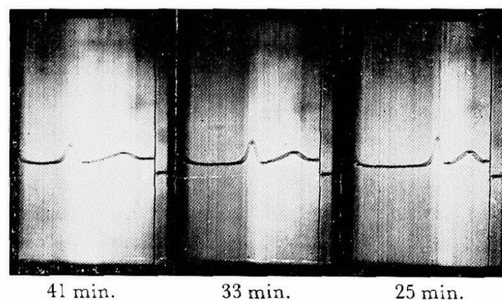


FIG. 1. Electrophoretic patterns of purified cytochrome b_1. The prepation used had an E_{280}/E_{415} ratio of 2.98. Protein concentration, 10.3 mg./ml. pH 6.5, 0.05 M phosphate buffer. 110 volts. 10 mA. Temperature, 20°C. a) left, ascending pattern, and right, descending pattern. b) ascending pattern.

Sedimentation Analysis — Although nearly homogeneous in electrophoresis, the purified preparation was separated into two main components, one colored and the other colorless, when subjected to ultracentrifugal analysis. From the sedimentation patterns shown in Fig. 2, the colored component (cytochrome b_1) was found to have a sedimentation constant of 20.3 S under the conditions employd, whereas that of the slowly sedimenting colorless component was estimated to be 7.3 S. By measuring the areas occupied by the two peaks in the sedimentation patterns, it was estimated that the ultracentrifugal purity of cytochrome b_1 in this preparation was about 50 per cent. Since the preparation used in the analysis had an E_{280}/E_{415} ratio of 2.98, it would be expected that an ultracentrifugally homogeneous preparation of the cytochrome shows the ratio of about 1.4.



41 min. 33 min. 25 min.

FIG. 2. Sedimentation patterns of purified cytochrome b_1. The same preparation as in Fig. 1 was used. Protein concentration, 10.3 mg./ml. pH 7.0, 0.05 M phosphate buffer. 47,050 r.p.m. Temperature, 15°C.

Absorption Spectra—The absorption spectra of the purest prepation of cytochrome b_1 obtained are shown in Fig. 3. The oxidized form showed, in addition to a peak at 280 mμ, a Soret band at 415 mμ and small shoulders at 524 mμ and 564 mμ. A broad band around 360 mμ was also observable. On reduction with dithionite the Soret peak was intensified and shifted to 427 mμ, and at the same time α-

and β-bands appeared at 559 mμ and 530 mμ respectively. This preparation showed an E_{280}/E_{415} ratio of 1.95 and, assuming the ratio of 1.4 for the pure preparation, the purity of this preparation was estimated to be about 70 per cent. The extinction coefficients at absorption maxima were calculated from Fig. 3 and found to have the following values (cm.$^{-1}$ mM^{-1} on protoheme basis): Reduced form, 24.4 (559 mμ), 14.2 (530 mμ), 147 (427 mμ); oxidized form, 8.8 (524 mμ), 109 (415 mμ). These values, except for that of the reduced α-band, are smaller than those obtained for the less purified preparation reported in the previous paper (*8*).

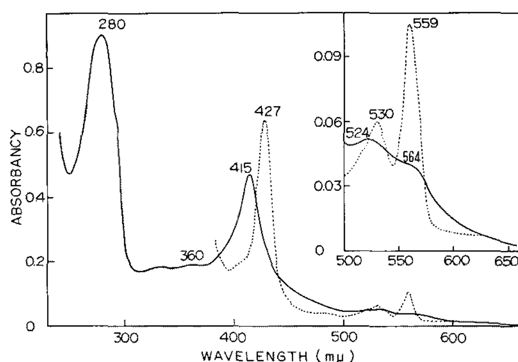


FIG. 3. Absorption spectra of purified cytochrome b_1. The preparation used had an E_{280}/E_{415} ratio of 1.95. Protein concentration, 0.96 mg./ml. pH 7.0, 0.05 *M* phosphate buffer. Optical path, 1.0 cm. ——, Oxidized form. ......, Reduced with $Na_2S_2O_4$.

Heme Moiety—The heme moiety of cytochrome b_1 has been shown to be protoheme (*8*). As reported on cruder preparations (*8*), the protoheme in the highly purified cytochrome underwent decomposition when the preparation was treated with *N* NaOH, 30% pyridine and a small amount of dithionite. The normal pyridine protohemochromogen spectrum could be obtained only by the procedure described by Appleby and Morton (*32*). The heme moiety could be easily split off from the protein by treating the cytochrome with acid acetone. Fig. 4 shows the spectrum of the heme thus extracted in acid acetone. This spectrum is identical with that of protohemin measured in acetone in the presence of hydrochloric acid (*32*).

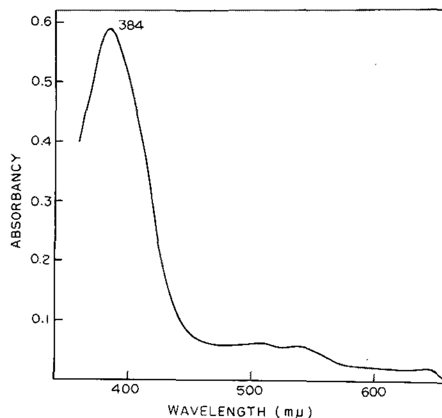


FIG. 4. Absorption spectrum of the heme moiety split from purified cytochrome b_1. Measured in acid acetone. The heme was extracted from about 30 mg. of the cytochrome preparation into 3 ml. of acid acetone.

Reconstruction from Apoprotein and Hemin—Even when the splitting with acid acetone was carried out at −50° to −60°C, the apoprotein recovered was very difficultly soluble in Tris-HCl buffer, pH 8.5, suggesting that it had suffered denaturation. The apoprotein was, however, completely devoid of heme as will be seen from Curves A and B of Fig. 5. When a solution of crystalline protohemin was added to a suspension of the apoprotein, the recombination was observed spectrophotometrically as shown in Curve C of Fig. 5. The reduced form of the reconstructed cytochrome b_1, however, showeed broader and more indistinct α- and β- bands as compared with those of original cytochrome. Furthermore, unlike native cytochrome b_1, the reduced form of the reconstructed cytochrome could combine with CO and the CO complex showed a sharp Soret peak at 422 mμ. As already reported (*8*), the same spectrum can be obtained when CO is added to cytochrome b_1 that had been denatured by heating or by aging. It is apparent that the reconstructed preparation represents denatured cytochrome b_1.

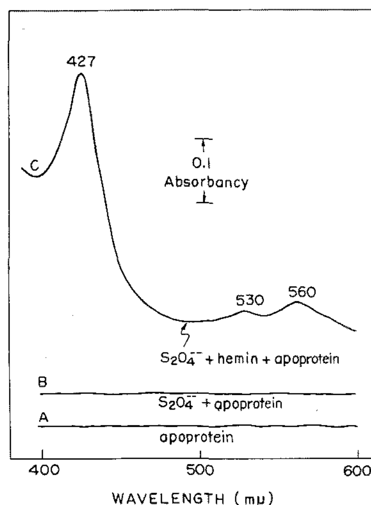


FIG. 5. Reconstruction of cytochrome b_1 from apoprotein and hemin chloride. 2 ml. of apoprotein suspension (pH 8.5) prepared as described in text and 0.1 ml. of 0.66 mM hemin solution (pH 8.0) were used. Curve A, apoprotein alone; Curve B, apoprotein reduced with $Na_2S_2O_4$; Curve C, apoprotein plus hemin reduced with $Na_2S_2O_4$.

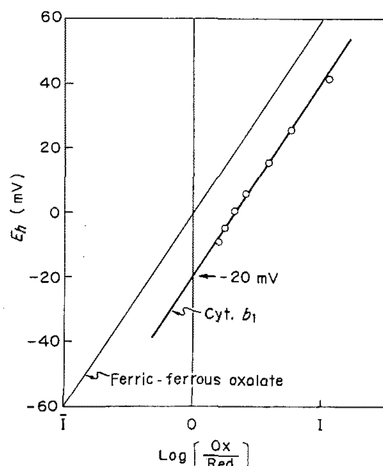


FIG. 6. Oxidation-reduction potential of purified cytochrome b_1. The preparation used had an E_{280}/E_{415} ratio of 2.98. For the experimental details see text.

Oxidation-Reduction Potential—The results of the spectrophotometric titration of purified cytochrome b_1 in the ferric-ferrous oxalate system are plotted in Fig. 6 according to the procedure of Hill (*23*). The normal oxidation-reduction potential (E_0') of the ferric-ferrous oxalated system at pH 7.0 was assumed to be zero as reported by Michaelis and Friedheim (*33*). As will be seen from Fig. 6, the E_0' value for cytochrome b_1 at pH 7.0 and 25°C was determined to be −20 mV. Three different determinations on the cytochrome preparations of different purity also gave values ranging from −15 mV to −25 mV.

Reactivity — Purified cytochrome b_1 was instantaneously and fully reducible by sodium dithionite and by sodium borohydride even in the presence of air. Under anaerobic conditions it was also rapidly reduced, though not fully, by reduced vitamin K_3 and by reduced UQ_8; the reduction by the latter could be observable only when the reagent was dispersed with the aid of 0.5 % deoxycholate. Ascorbate (0.01 M) could slowly reduce the cytochrome in the absence of oxygen; the reduction was faster at pH 8.0 than at pH 6.5. Cysteine (0.01 M) also reduced the cytochrome anaerobically, but the rate of reduction was very low. The cytochrome could not be reduced both by NADH and NADPH. The reduced form of cytochrome b_1 was fairly strongly autoxidizable and this autoxidation was insensitive to 0.01 M cyanide or azide. Although mammalian cytochrome c could be slowly reduced by formate in the presence of the particulate fraction of *E. coli*, no interaction could be observed between the reduced form of purified cytochrome b_1 and the oxidized form of mammalian cytochrome c. The purified cytochrome, both in the reduced and oxidized forms, could bind neither cyanide, CO, nor ethyl isocyanide. When the cytochrome was denatured by heating at 60°C for 15 minutes or by subjecting it to repeated freezing and thawing, however, the cytochrome, when reduced, became capable of combining with CO and ethyl isocyanide as evidenced by the sharpening and shift of the Soret band to 422 mμ and 430 to 432 mμ respectively.

Stability—The absorption spectra of both oxidized and reduced forms of purified cytochrome b_1 remained unaffected by changing the pH from 5.0 to 11.0. Even at pH 4.0,

where the cytochrome precipitates, the dithionite-reduced minus oxidized difference spectrum of the preparation was essentially normal. However, the CO-difference spectrum of the reduced cytochrome at pH 4.0 showed the Soret peak at 420 mμ, indicating the emergence of CO-binding capacity. In the region from pH 5.0 to 9.0, on the other hand, CO had no effect on the spectrum of the reduced cytochrome. Bubbling CO into the reduced cytochrome solution at pH 10.0 and 11.0 caused a decrease in absorbancy at 427 mμ, the Soret peak of the reduced form, but no shift of the peak was observed.

Although the oxidized form was denatured by heating at 60°C for 7 minutes, the cytochrome reduced with a small amount of dithionite was stable when heated at 60°C for 7 minutes under pure nitrogen. The preparation thus treated showed the normal spectrum and did not combine with CO. No changes in absorption spectra were observed when the preparation was kept at 0°C for 4 to 5 days in 0.05 *M* phosphate buffer, pH 7.0. It was also stable for at least two weeks when stored at −20°C.

Contaminating Enzymes — Neither NADH nor NADPH could be oxidized in the presence of purified preparations of cytochrome b_1. They were also free from the activities of succinate and glucose dehydrogenases. A very low, but definitely detectable, activity of formate dehydrogenase was still present in all the purified preparations. Nitrate reductase was absent from most of the preparation, but a trace of the activity could be detected in a few preparations of low purity.

Chemical Constituents — The purified preparation of cytochrome b_1 having an E_{280}/E_{415} ratio of 2.98 contained 3.2 mμmoles of protoheme per mg. of protein. Since the ultracentrifugal purity of this preparation was about 50 per cent, the heme content of pure cytochrome b_1 was assemed to be about 6 mμmoles per mg. of protein. It was further revealed that the same prepation contained 60 mμatoms of iron and 0.2 mμmoles of flavin per mg. of protein. However, it is not yet possible to determine whether or not these constituents are associated with the cytochrome molecule. It seems likely that the contaminating formate dehydrogenase is responsible for the small amount of flavin detected. Although the absorption spectrum of this preparation showed no shoulder at 260 mμ, chemical analysis revealed the presence of 31 μg. of RNA per mg. of protein. However, no DNA could be detected. Deoxycholate which had been used for the solubilization was still present in the preparation at a concentration on 15 mμmoles per mg. of protein, or about 5 moles per mole of protoheme.

DISCUSSION

Cytochrome b_1 of *E. coli* is exclusively localized in the particulate cell fraction and has only recently been obtained in soluble state (*6—8*, *10*). The soluble preparations so far reported are, however, rather crude and in most cases are inseparably associated with other enzymatic entities. In the present work it was for the first time possible to purify the cytochrome to such an extent that the characterization of some of its physicochemical properties was amenable.

The purified cytochrome, though still contaminated by a colorless impurity, had a a sedimentation constant of 20.3 S. This value suggested that cytochrome b_1 has a molecular weight of the oder of 600,000 to 800,000. On the other hand, a preparation of about 50 % purity was shown to contain 3.2 mμmoles of protoheme per mg. of protein. From this figure it is possible to calculate a minimum molecular weight of about 160,000 for the cytochrome. It appears, therefore, that the cytochrome contains 4 to 5 heme groups per molecule. However, it is equally probable that the cytochrome contains only one heme per molecule, but exists as tetra- or pentamer in solution. At present it is not yet possible to decide which is really the case, but it is interesting to recall that the mitochondrial bound cytochromes of mammalian origin, *i. e.* cytochromes *b* (*34*), c_1 (*35*) and *a* (*36*), have been reported to exist as polymerized forms when solubilized and highly purified.

Bacterial cytochromes having spectral pro-

perties characteristic of cytochrome b_1 have also been isolated and partly purified by Vernon (*37*, *38*) from *Micrococcus denitrificans*, *Pseudomonas denitrificans* and an unidentified pseudomonad, and by Jackson *et al.* (*39*) from *Micrococcus lysodeikticus*. The reported properties of these pigments are similar to those of *E. coli* cytochrome b_1 not only in absorption spectra but also in autoxididizability, acidic nature, *etc*. However, these preparations seem to have been much less purified than that reported here. Furthermore, it is not yet certain whether or not they are firmly bound to the particulate cell fractions as in *E. coli*. The procedures employed for their isolation rather suggest that the cytochromes in *M. denitrificans* and *P. denitrificans* are present mainly in the soluble cytoplasmic fraction. They seem to further differ from the *E. coli* cytochrome in that the former have been reported to be oxidizable directly by nitrate (*37*), whereas a a specific enzyme nitrate reductase is required for the oxidation of the latter by nitrate (*11*—*16*). It appears very likely that, despite their spectral identity, the *E. coli* pigment differs from the *Micrococcus* and *Pseudowonas* cytochromes in their intracellular localization and probably also in their properties and functions. In this connection, it is interesting to note that a solubilized form of a CO-binding cytochrome recently discovered in mammalian liver microsomes shows absorption spectra closely similar to those of cytochrome b_1 (*40*, *41*).

SUMMARY

Cytochrome b_1 was solubilized from the particulate fraction of *Escherichia coli* with the aid of snake venom and deoxycholate and was extensively purified by ammonium sulfate fractionation and hydroxylapatite column chromatography. The purifed preparation was still contaminated by a colorless impurity which sedimented more slowly than the cytochrome in the ultracentrifugal field; the purity being estimated to be 50 to 70 per cent. A molecular weight of 600,000 to 800,000 could be expected for the cytochrome from its sedimentation constant. On the other hand, a minimum molecular weight of about 160,000 was obtained from the heme content of the cytochrome. The cytochrome could be split by acid acetone into protoheme and an apoprotein moiety. The recombination of the two components could be observed spectrophotometrically, but the reconstructed cytochrome was no more native as evidenced by its capabiliiy to combine with CO. The normal oxidation-reduction potential of purified cytochrome b_1 was determined to be about -20 mV at pH 7.0 and 25°C. Spectral properties, reactivity and chemical composition of the purified preparation were also studied.

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