The Journal of Biochemistry, Vo. 52. No. 2, 1962

Solubilization and Properties of Formate Dehydrogenase and Cytochrome b_1 from *Escherichia coli**

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(Received for publication. May, 11, 1962)

Dehydrogenation of formate by *Escherichia coli* has been studied with intact cells and cell-free extracts (*1—3*). However, the nature of formate dehydrogenase of this organism has remained largely unelucidated because of its firm association with the particulate fraction of the cell.

On the other hand, it has been anticipated from the study of nitrate respiration that formate is a very effective and important source of hydrogen for respiratory reduction of nitrate in *E. coli*. It has also been shown that the catalytic system responsible for the formate-nitrate reaction resides on the particulate fraction (*4*). It has further been established that this system consists of at least three components, *i. e.* formate dehydrogenase, cytochrome b_1 and nitrate reductase. Taniguchi and Itagaki (*5*) have solubilized and highly purified nitrate reductase and showed that this enzyme is a metalloprotein containing molybdenum and non-heme iron. The characterization of the other two components have, however, not yet been accomplished.

Recently, Wrigley and Linnane (*6*) have briefly reported the solubilization and partial purification of a formate dehydrogenase-cytochrome b_1 complex from aerobically grown cells of *E. coli*. This complex is reported to be free from nitrate reductase and its cytochrome moiety to be readily reducible by formate. We have also independently succeeded in solubilizing formate dehydrogenase from the particulate fraction of *E. coli* grown anaerobically under conditions favorable for nitrate respiration. This preparation contained not only cytochrome b_1 but also nitrate reductase in addition to formate dehydrogenase. It also differed from the Wrigley-Linnane complex in that the former had lost a factor necessary for the reduction of cytochrome b_1 by formate.

This paper describes the solubilization and partial purification of a preparation containing formate dehydrogenase, cytochrome b_1 and nitrate reductase from *E. coli* grown under the conditions of nitrate respiration. It also reports some properties of formate dehydrogenase and cytochrome b_1 in this preparation. A preliminary account of this work has been published (*7*).

EXPERIMENTAL

Bacterial Cells—E. coli, Yamagutchi strain, was grown in a medium containing the following per liter: polypeptone (Wako), 10g.; KNO_3, 3.5g.; sodium formate, 3.5 g.; K_2HPO_4, 2 g.; casamino acids (Difco), 1 g.; yeast-extract powder (Daigo), 1 g.; glucose, 20 g.; pH adjusted to 7.2 with NaOH. The cells grown aerobically on a peptone-meat extract-agar medium were collected from two Roux bottles and inoculated to 6 liters of the above medium filled to the top of large Erlenmeyer flasks. The flasks were allowed to stand 37°C for 5—6 hours. During cultivation the medium was maintained under practically anaerobic conditions owing to powerful respiration of the growing cells and to very slow diffusion of oxygen into the medium. The cells were harvested and washed with cold water in a Sharples centrifuge until the washing became free from nitrite. About 15 g. of wet cells were obtained from 6 liters of medium.

Particulate Preparation—The washed cells were disrupted and the particulate fraction was prepared therefrom as described by Taniguchi and Itagaki (*5*). All the preparative manipulations were carried out at 4°C unless otherwise stated.

Solubilization—The washed particulate fraction

* This paper is dedicated to Prof. H. Tamiya on the occasion of his 60th birthday.

was suspended at a final concentration of 1—1.5 mg. nitrogen per ml. in a medium containing 0.1% crude snake venom (*Trimeresurus flavoviridis*), $10^{-5}M$ ethylenediamine tetracetate and 0.05 *M* Tris-HCl buffer (pH 8.0). The mixture was incubated at 0°C for 16—18 hours in a nitrogen atmosphere, and then centrifuged for 60 minutes at 78,000×*g* in a Hitachi preparative ultracentrifuge. The supernatant was used for purification.

Purification of Formate Dehydrogenase—To the yellowish supernatant obtained above was added saturated ammonium sulfate solution (previously neutralized with NH_4OH) to 45% saturation under a stream of nitrogen. After standing for 20 minutes, the mixture was centrifuged at 15,000×*g* for 15 minutes and the precipitate discarded. The concentration of ammonium sulfate in the supernatant was then raised to 60% saturation and the resultant precipitate was collected by centrifugation at 78,000×*g* for 30 minutes. The reddish brown precipitate was dissolved in 0.05 *M* Tris-HCl buffer (pH 7.1) corresponding to one third volume of the original supernatant. To this solution was added one fifth volume of fresh calcium phosphate gel (about 50 mg. dry weight per ml.) prepared according to Keilin and Hartree (*24*). The gel was recoverd by centrifugation and washed with 10 volumes of 0.05 *M* Tris-HCl (pH 7.1). The enzyme was then eluted from the gel by two successive treatments with small amounts of 0.2 *M* citrate-phosphate (pH 7.1). The eluate thus obtained was reddish amber in color and contained cytochrome b_1 and nitrate reductase in addition to formate dehydrogenase.

Assay for Formate Dehydrogenase—Four different methods were employed.

1) *Nitrate reductase method*: The activity of formate dehydrogenase was measured in this method by linking it to an excess of nitrate reductese with the aid of methylene blue and by determining the nitrite formed by this reconstructed system. In the main compartment of a Thunberg tube were placed 50 μmoles of Tris-HCl buffer (pH 7.1), formate dehydrogenase-free nitrate reductase (20—40 units) prepared from *E. coli* through the gel adsorption step according to Taniguchi and Itagaki (*5*), and formate dehydrogenase samples (5—10 units) dissolved in 0.05 *M* Tris-HCl buffer (pH 7.1) containing 0.5 mg. bovine serum albumin (Armour) per ml. and 5 m*M* glutathione. The side compartment received 10 μmoles KNO_3, 1 μmole methylene blue and 10 μmoles sodium formate. The total volume was 2.0 ml. After evacuation, the tube was preincubated for 5 minutes at 37°C and the reaction was started by tipping. After 10 minutes reaction at 37°C, 1.0 ml. of reaction mixture was pipetted out and added to 1.0 ml. of 0.05 *M* $CdSO_4$ solution for deproteinization. The removal of methylene blue and the determination of nitrite were carried out as described by Itagaki and Taniguchi (*8*).

2) *Manometric method*: The anaerobic production of CO_2 from formate in the presence of methylene blue or other dyes was measured as described by Peck and Gest (*2*).

3) *Spectrophotometric method*: The rate of dye reduction by formate was spectrophotometrically measured in a Cary recording spectrophotometer at room temperature. The reaction was run in Thunberg-type cuvettes (optical path, 1.0 cm.) under anaerobic conditions. The reaction mixture contained in a final volume of 3.0 ml. the following: 0.05 *M* Tris-HCl buffer (pH 7.1), 1.0 μmoles sodium formate, and either one of the three dyes, 2, 6-dichlorophenol indophenol (156 mμmoles), methylene blue (81 mμmoles) and neotetrazolium chloride (82 mμmoles). The wavelengths selected were 600, 650 and 545 mμ for indophenol, methylene blue and neotetrazolium, respectively.

4) *Neotetrazolium method*: Although neotetrazolium in a poor hydrogen acceptor for the formate enzyme, this was used for studying the effect of oxygen on the enzyme activity, since the reduced dye (diformazan) is not autoxidizable. The reaction mixture contained 82 mμmoles neotetrazolium chloride, 10 μmoles sodium formate, 50 μmoles Tris-HCl buffer (pH 7.1) and enzyme in a final volume of 2.0 ml. The reaction was carried out in Thunberg tubes which had been evacuated and flashed with oxygen-free nitrogen three times. After preincubation for 5 minutes, the reaction was started by tipping formate in the side arm into the main compartment. After 10 minutes reaction at 37°C, 2.0 ml. of ethyl acetate and a small amount of NaCl were added to the mixture followed by vigorous shaking. The mixture was then centrifuged at 3,000 r.p.m. for 5 minutes and the pink color of the ethyl acetate layer (due to diformazan) was measured at 545 mμ in a Coleman spectrocolorimeter. The concentration of neotetrazolium chloride was determind at 545 mμ after converting the dye into diformazan by the addition of dithionite.

5) *Enzyme unit*: One unit of formate dehydrogenase was defined as the amount of enzyme which produces 1 μmole of nitrite per hour under the conditions of nitrate reductase method. Specific activity was expressed as units per mg. nitrogen as determined by the Kjeldahl method.

Assay for Nitrate Reductase—This was performed with reduced methyl viologen as hydrogen donor according to the method of Taniguchi and Itagaki (*5*).

Determination of Protoheme and Cytochrome b_1—Protoheme was converted to pyridine hemochromogen by the method of Appleby and Morton (*9*) and determined spectrophotometrically using a millimolar extinction coefficient value of 32.5 at 557 mμ. This value was determined with pyridine hemochromogen prepared by the Appleby-Morton method from crystalline protohemin. Cytochrome b_1 was determined from absorption spectra of dithionite-reduced samples. The millimolar extinction coefficients at 559 or 415 mμ recorded in Table IV were employed.

Detection of Flavin—Flavin was detected fluorometrically by the lumiflavin method (*10*). Riboflavin purified by paper chromatography was used as the standard. The fluorescence spectra was measured in an Aminco recording spectrofluorometer using cells of 1.0 cm. optical path. The sample were excited by the light of 365 mμ.

RESULTS

Effect of Medium Composition

The levels of formate dehydrogenase and cytochrome b_1 in anaerobically grown cells of *E. coli* were found to be considerably affected by the con- centration of both nitrate and formate in the medium. The highest production of these enzymes was observed when 0.3—0.5 per cent each of nitrate and formate were present in the medium. Such conditions which are favorable for nitrate respiration also shorten- ed the lag period of growth and the cells harvest ed from these cultures were most reddish in color. If nitrate was withdrawn from the medium, the synthesis of formate dehydrogenase was decreased to a very low level in spite of sufficient supply of formate during the growth. In a medium free from both nitrate and formate the cells produced less than one tenth of cytochrome b_1 as compared with those in enriched media. It is obvious that the conditions of nitrate respiration are associated with high synthesis of formate dehydrogenase as well as that of cytochrome b_1.

The cells anaerobically grown in the presence of both nitrate and formate were further found to contain succinate dehydrogenase in low concentrations; its activity being less than 10 per cent of that of formate dehydrogenase. The activities of DPNH, TPNH and glucose dehydrogenases were still lower. Although *E. coli* has been reported to possess high activities of formate hydrogenlyase and hydrogenase, these activities were scarcely detectable in the cells obtained.

Solubilization and Purification

The treat- ment of the particulate fraction from *E. coli* with snake venom and deoxycholate could solubilize the formate dehydrogenase activity in a form not sedimentable by centrifugation at $105{,}000 \times g$ for 60 minutes. The presence of ethylenediamine tetracetate as well as the absence of oxygen during the treatment increased the yield of soluble enzyme. A standard solubilization procedure as described in "Experimental" was devised based on the results of a series of preliminary experiments. The recovery of formate dehydrogenase in the $78{,}000 \times g$ supernatant was usually 60—90% according to this procedure, which also resulted in the solubilization of considerable amounts of cytochrome b_1 and nitrate reductase. However, neither cytochromes a_1 and a_2 nor dehydrogenases for DPNH, TPNH, succinate and glucose were detectable in the supernatant.

Digestion of the particulate fraction with chymotrypsin and crude pancreatic lipase ("steapsin"), and treatments with sodium dodecyl sulfate, Tween 80, Emasol* and Emalgen** either inactivated or brought about only insufficient solubilization of formate dehydrogenase. The enzyme could, however, be relatively effectively, though not satisfactorily, solubilized by digesting the particles with heat-treated snake venom at pH 9.0.

The solubilized formate dehydrogenase could be further purified by ammonium sulfate fractionation and calcium phosphate gel treatment as described in "Experimental." Since the soluble enzyme was relatively labile to oxygen, the ammonium sulfate fractionation was performed under anaerobic conditions. The partially purified preparation finally obtained was reddish amber in color and represented a 9—10 fold purification over the particulate preparation. A summary of

* Polyoxyethylene-sorbitan mono-oleate

** Polyoxyethylene-octyl phenol-ether

TABLE I

Summary of Solubilization and Purification of Formate Dehydrogenase, Cytochrome b_1 and Nitrate Reductase from E. coli Particles

Purification step	Formate dehydrogenase		Nitrate reductase		Cytochrome b_1		Total nitrogen mg.
	units	u./mg. N	units	u./mg. N	mμmoles	mμmoles /mg. N	
Particulate fraction	35,000	74	82,500	174	1,620	3.42	474
Solubilized supernatant	32,000	139	41,000	178	1,090	4.72	230
Ammonium sulfate fraction	18,000	323	45,600	777	661	11.3	58.6
Gel eluate	8,160	790	17,900	1,740	227	22.0	10.3

90 g. of wet cells were used. Formate dehydrogenase was assayed by the nitrate reductase method.

TABLE II

Acceptor Specificity of Formate Dehydrogenase

Acceptor	E'_0, (pH 7) volts	Relative formate dehydrogenase activity	
		for purified preparation	for particulate preparation
Methylene blue	+0.011	100	100
Toluidine blue	+0.011	100	64
Thionine	+0.051	24	40
Nile blue	−0.116～−0.150	81	8
Janus green	−0.256	111	34
2,6-Dichlorophenol indophenol	+0.217	0	51
Benzyl viologen	−0.363	26	—
Methyl viologen	−0.446	0	0
Phenazine methosulfate	+0.080	263	296
Ferricyanide	+0.360	11 100[a]	— 100[a]
Neotetrazolium chloride		1	1

All the acceptors were tested at a concentration of 5×10^{-3} *M*. The assay of formate dehydrogenase activity was performed by the manometric method.

a) 2.5×10^{-2} *M*

the purification procedure is shown in Table I. It will be seen that the purification procedure almost equally enriched formate dehydrogenase, cytochrome b_1 and nitrate reductase.

Properties of Formate Dehydrogenase

Kinetic Properties—When the purified soluble preparation was assayed by the manometric method using methylene blue as acceptor, it produced 15—20 μmoles CO_2 from formate per minute per mg. nitrogen, a value roughly equivalent to 1,000 units per mg. nitrogen. The optimum pH in Tris-HCl buffer was 7.1; the activity was somewhat lower in phosphate buffer. The apparent Michaelis constants for formate and methylene blue were 1.3×10^{-4} *M* and 2×10^{-4} *M*, respectively. The activity of the particulate preparation was stable at least for two weeks when stored in a deep freeze, whereas that of purified enzyme was 30 per cent inactivated during 4 days in a frozen state. The latter was, however, inactivated to only a small extent when kept at 4°C for two days.

Acceptor Specificity—Table II shows that among the redox dyes tested phenazine methosulfate is the most effective acceptor for both the particulate and solubilized forms of formate dehydrogenase. Although we have

preliminarily reported that this dye was inactive as acceptor (7), it was later found that this erroneous observation had been due to the use of low-quality samples of the dye in earlier experiments.

It should be noted that 2, 6-dichlorophenol indophenol is inactive as acceptor for the solubilized enzyme, though it is a fairly good acceptor for the particulate system. This fact is more clearly illustrated in Fig. 1 in which the reduction by the two types of preparations of methylene blue and the indophenol dye were spectrophotometrically followed. It is conceivable that a factor (or factors) required for the indophenol reduc- tion has been lost during the solubilization and/or purification. DPNH, TPNH and mammalian cytochrome *c* were unable to act as acceptors for the solubilized enzyme. No simple relations seem to exist between the normal redox potentials of dyes and their efficiencies as acceptors.

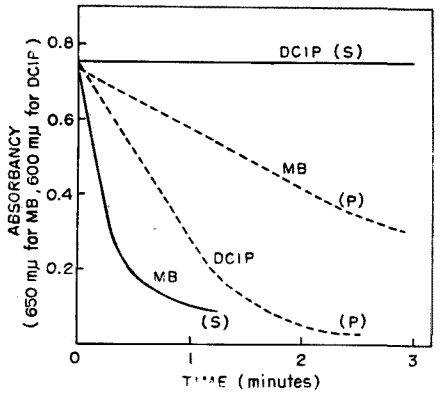

FIG. 1. Methylene blue and 2, 6-dichlorophenol indophenol as acceptors for particulate and solubilized formate dehydrogenase preparations. The reaction mixture contained in a final volume of 3.0 ml: 81 mμmoles methylene blue or 156 mμmoles 2, 6-dichlorophenol indophenol; 10 μmoles sodium formate; 50 μmoles Tris-HCl buffer (pH 7.1); and enzyme (24 μg. N particulate preparation or 11.8 μg. N solubilized preparation). The reaction was carried out anaerobically in Thunberg-type cuvettes at room temperature. MB, methylene blue; DCIP, 2, 6-dichlorophenol indophenol; P, particulate preparation; S, solubilized preparation. The absorbancy changes were followed at 650 mμ for MB, and at 600 mμ for DCIP.

Effects of Inhibitors—The effects of various types of inhibitors on solubilized formate dehydrogenase were examined manometrically using methylene blue as acceptor. As shown in Table III, the enzyme was strongly sensitive to cyanide. The other metal-binding reagents such as α, α'-dipyridyl and *o*-phenanthroline also exerted inhibitions, though not as remarkably as cyanide. Furthermore, two typical inhibitors for sulfhydryl enzymes, *p*-chloromercuribenzoate and monoiodoacetate, and an inhibitor for flavin enzymes, atebrin, were also shown to be inhibitory. It was suggested that a metal, sulfhydryl groups and flavin may be involved in the activity of formate dehydrogenase.

Presence of Flavin—The involvement of flavin received further support from its fluorometric detection in the purified preparation. Fig. 2 shows that the fluorescence spectrum of the purified sample processed according to the lumiflavin procedure has a maximum at 530 mμ when excited by the light of 365 mμ. This position of the maximum coincides with that of lumiflavin prepared from an authentic

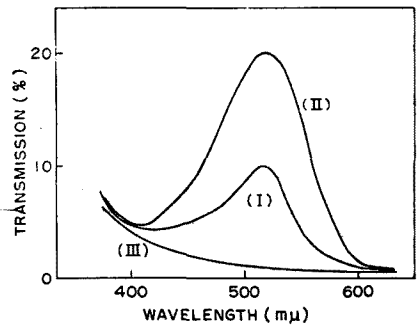

FIG. 2. Fluorescence spectrum of lumiflavin prepared from purified formate dehydrogenase preparation. Lumiflavin was prepared as follows: To 1.0 ml. sample solution (1.25 mg. *N*) was added 2.0 ml. of *N* NaOH and the mixture was irradiated with a UV-lamp for 20 minutes. After irradiation, 0.2 ml. of conc. acetic acid was added and lumiflavin was extracted with 5.0 ml. of redistilled chloroform. The spectra were obtained with activating light at 365 mμ. (I) purified formate dehydrogenase, (II) riboflavin control (1.2×10^{-4} m*M*), and (III) reagent blank.

TABLE III

Effect of Inhibitors of Purified Formate Dehydrogenase

Inhibitor	Concentration $M \times 10^3$	Inhibition %
KCN	5	71
NaN_3	5	35
o-Phenanthroline	2.5	38
α, α'-Dipyridyl	2.5	35
8-Hydroxyquinoline	1.25	9
p-Chloromercuribenzoate	1.25	46
Monoiodoacetate	5	42
N-Ethylmaleimide	2.5	3
Atebrin	5	65
2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide	(4.4×10^{-6} *M*)	0
Dicumarol	1.25	32
Amytal	5	35
Hypophosphite	5	26
Formaldehyde	5	26
Formamide	5	26

The formate dehydrogenase assay was performed by the manometric method.

sample of riboflavin. The absorption spectrum of the purified enzyme (Fig. 5) also showed a shoulder around 455 mμ which disappeared on addition of a small amount of formate plus vitamin K_3 or dithionite.

Inhibition by Oxygen—The reduction of neotetrazolium chloride by the purified enzyme was found to be considerably inhibited under aerobic conditions.

As shown in Fig. 3, the reduction of this dye catalysed by the purified enzyme was very slow under atmospheric oxygen. The reduction rate was, however, increased remarkably when the reaction was carried out in the complete absence of oxygen. Fig. 4 further shows that the reaction was inhibited by 50 per cent in the presence of 5% (vol.) oxygen in the atmosphere. Nearly complete inhibition was caused by 50% (vol.) oxygen. Similar, but less pronounced, inhibitions by oxygen was also observed by the manometric method using the dyes other than neotetrazolium chloride as acceptors. The inhibition is reversible and disappears immediately on removal of oxygen from the reaction system.

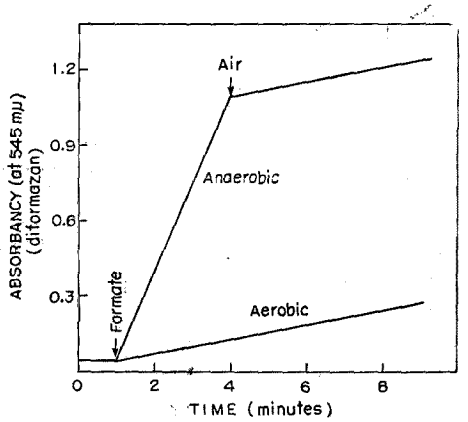

FIG. 3. Inhibition by aerobiosis of solubilized formate dehydrogenase. The reaction mixture consisted of 82 mμmoles neotetrazolium chloride, 10 μmoles sodium formate, 50 μmoles Tris-HCl buffer (pH 7.1), and 62 μg. N of purified enzyme preparation in a final volume of 2.0 ml. Anaerobic experiments were performed in Thunberg-type cuvettes, whereas aerobic runs in usual open cuvettes. Room temperature.

Properties of Cytochrome b_1

Fig. 5 shows the absorption spectra of the purified enzyme preparation. It is evident from characteristic peaks at 559 (α), 530 (β) and 427 mμ (Soret) of the dithionite-reduced spectrum that this preparation contains cytochrome b_1. Correspondingly, the oxidized spectrum has a Soret peak at 415 mμ together

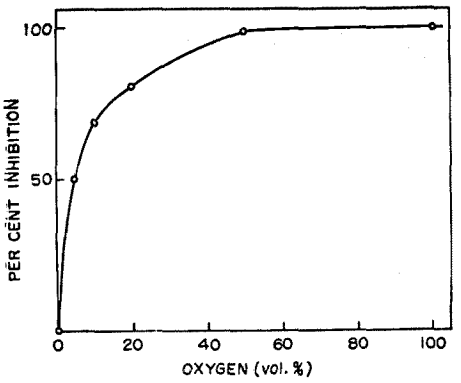

FIG. 4. Effect of oxygen tension on formate dehydrogenase activity. The reaction was carried out in Thunberg tubes filled with an oxygen-nitrogen mixture; the volume percent of oxygen being indicated on the figure. Other conditions were the same as for Fig. 3. The formation of diformazan was followed by extracting it into ethylacetate and measuring at 545 mμ. Degrees of inhibition were calculated based on the rate obtained in the complete absence of oxygen.

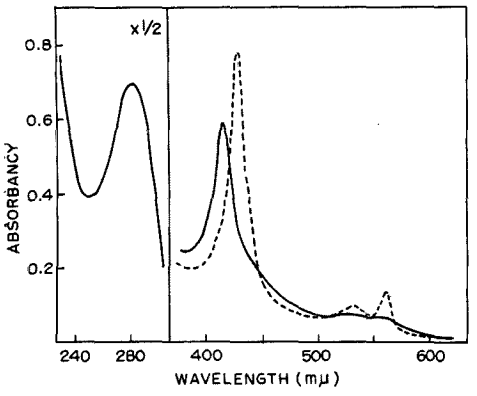

FIG. 5. Absorption spectra of oxidized and dithionite-reduced preparation of purified formate dehydrogenase. 5.8 mg. protein of sample was dissolved in 0.05 *M* Tris-HCl buffer, pH 7.1. —— Oxidized form; ····· Reduced form.

with a small peak at 524 mμ and a shoulder at 564 mμ. When converted to reduced pyridine hemochromogen by the method of Appleby and Morton (*9*), the preparation showed a spectrum with maxima at 557, 525 and 418 mμ. The positions of these peaks and the general shape of spectrum were identical with those of the reduced pyridine hemochromogen prepared from crystalline protohemin. Furthermore, the heme could be readily split off from the protein when treated with acid acetone or acid methyl-ethyl ketone. It seems that the heme is present in this preparation in a somewhat labile configuration, since the treatment of the preparation with *N* NaOH, 30% pyridine and dithionite (a procedure most commonly used for preparing reduced pyridine hemochromogen) gave rise to a decomposed product whose spectrum was quite different from that of reduced pyridine protohemochromogen.

The protoheme content of the purified preparations ranged from 1 to 3 mμmoles per mg. protein. Assuming that the cytochrome contains one protoheme per mole, the molecular extinction coefficients of the reduced cytochrome were calculated and the values at its α, β and Soret peaks are recorded in Table IV together with corresponding values for mammalian cytochrome *b* (*11*) and yeast cytochrome b_2 (*9*). It will be seen that the values for cytochrome b_1 are intermediate between those of cytochromes *b* and b_2.

Carbon monoxide, cyanide and ethyl isocyanide had no effect on the spectra of oxidized and reduced cytochrome b_1 in fresh preparations. However, repeated freezing and thawing for about 10 days or treatment at 60°C for 10 minutes rendered the reduced cytochrome capable of binding carbon monoxide and ethyl isocyanide as evidenced by the shift of Soret peak from 427 mμ to 422 and 430 mμ, respectively. Besides dithionite, formate plus vitamin K_3 could fully reduce the cytochrome as described below. The reduced form of cytochrome b_1 thus obtained was rapidly reoxidizable on exposure to oxygen (Fig. 6). This reoxidation was not inhibibited by $10^{-2}M$ cyanide or azide indicating the autoxidizability of the cytochrome.

Link between Formate Dehydrogenase and Cytochrome b_1

When formate was added to the particulate preparation, cytochrome b_1 was rapidly and fully reduced. Similarly, the

TABLE IV

a) Millimolar Extinction Coefficients of Purified Cytochromes b (11), b_1 and b_2 (9)

Cytochrome b_1		Cytochrome b		Cytochrome b_2	
Absorption maxima (mμ)	E_{mM}	Absorption maxima (mμ)	E_{mM}	Absorption maxima (mμ)	E_{mM}
(Reduced form)					
559 (α)	24.4	564	20.8	557	38.8
530 (β)	16.1	530	13.9	528	18.6
427 (Soret)	154	430	135	423	171
(Oxidized form)					
564 (shoulder)	—				
524	15.2	525–556	—	530–556	—
415	135.2	416	118	413	137

b) Spectrophotometric Data for Purified Cytochrome b_1

1. Millimolar extinction coefficient increments:
   ΔE_{mM} 559 mμ (reduced) minus 542 mμ (oxidized) = 16.1
   ΔE_{mM} 427 mμ (reduced) minus 410 mμ (oxidized) = 153
2. Absorbancy ratios:
   $E_{559}/E_{530}=1.52$; $E_{427}/E_{559}=6.3$; $E_{427}/E_{530}=9.6$; $E_{427}/E_{415}=1.14$.

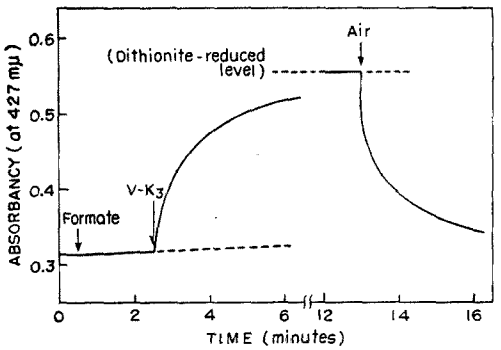

FIG. 6. Reduction and reoxidation of cytochrome b_1 in purified formate dehydrogenase preparation. Reduction phase was carried out anaerobically in a Thunberg-type cuvette. To 1.9 ml. mixture containing 3.8 mg. protein were added 5 μmoles sodium formate (0.05 ml.) and 75 mμmoles vitamin K_3 (0.05 ml. in 50% aqueous acetone) as indicated. Room temperature, pH 7.1 (Tris-HCl final concentration 0.10 *M*).

cytochrome in the Wrigley-Linnane solubilized preparation is reported to be reducible by formate alone (*6*). However, the cytochrome in the purified preparation obtained in this study could be reduced by formate only at negligibly slow rates or sometimes could not be reduced at all in spite of a powerful formate dehydrogenase activity of the preparation. It was suggested that a factor is required for this reduction and this factor has been lost in the purified preparation. It was, in fact, found that the reduction of cytochrome b_1 by formate could be greatly enhanced by the addition of a small amount of vitamin K_3; the cytochrome being fully reduced under anaerobic conditions (Fig. 6). In preliminary experiments it was further disclosed that a lipid fraction extracted from the particulate fraction was capable of replacing vitamin K_3 in this linking role if the lipid was dispersed with the aid of suitable detergents.

DISCUSSION

The synthesis of formate dehydrogenase by *E. coli* during anaerobic growth was shown to be more profoundly increased by nitrate rather than by formate. The presence of nitrate in the medium also stimulated the production of cytochrome b_1. It was further found that the cells adapted to nitrate respiration are relatively deficient in dehydrogenases acting on DPNH, TPNH, succinate and

glucose. These findings suggest that formate dehydrogenase as well as cytochrome b_1 are closely connected to the mechanism of nitrate respiration. It is of interest in this connection that *E. coli* produces large quantities of fomate when anaerobically grown in the presence of glucose. It has also been noted that *E. coli* grown anaerobically has higher activities of formate dehydrogenase than that grown aerobically. Formate has further been shown to be the most effective hydrogen donor for respiratory nitrate reduction in a halotolerant micrococcus,* although succinate is more suitable for nitrite reduction.

Formate dehydrogenase of *E. coli* is exclusively localized in the particulate fraction of the cell. Although numerous attempts have been mede to solubilize this enzyme with proteolytic and lipolytic enzymes, organic solvents or detergents, none of them have so far been successful except for the recent work of Wrigley and Linnane who obtained a soluble formate dehydrogenase-cytochrome b_1 complex by means of deoxycholate and ammonium sulfate (*6*). In the present work, it was independently possible to solubilize a preparation containing not only formate dehydrogenase and cytochrome b_1 but also nitrate reductase by digesting the particulate fraction with snake venom and deoxycholate under anaerobic conditions. The three components thus solubilized could be partially purified to similar extents by the same purification procedures. This fact suggests that they are located on the same fragments of the original particles. It is, however, premature to draw final conclutions on this point.

The association of *b*-type cytochromes with partially purified dehydrogenases is not uncommon. Among microbial enzymes, succinate dehydrogenase of *Propionibacterium* (*12*) and *Corynebacterium* (*13*), pyruvate oxidase of *E. coli* (*14*), lactate dehydrogenase of yeast (*9*), glucose dehydrogenase of *Bacillus anitratum* (*15*), etc. have been purified as complexes with *b*-type cytochromes. It is noteworthy that these enzymes, with possible exception of the last mentioned, are all of flavo- or metalloflavoprotein nature. The present investigation also provided evidence that formate dehydrogenase of *E. coli* belongs to the same category.

Cytochromes *b* (*11*) and b_2 (*9*) have been highly purified from heart muscle and yeast, respectively, and their properties well characterized. However, cytochrome b_1 has not yet been solubilized and, therefore, its properties have so far been studied only with the particulate-bound form. The solubilization and partial purification in the present study of a preparation containing this cytochrome has for the first time permitted closer examination of its properties and led to the decisive identification of its prosthetic group as protoheme and to the demonstration of its strong autoxidizability. It is, however, desirable to accomplish further purification in order to obtain more precise information concerning the nature of this important hemoprotein.

Although it has long been believed that formate dehydrogenase of *E. coli* can directly reduce cytochrome b_1 (*4*), the evidence presented in this paper clearly indicates the requirement of an additional factor for this reaction. It was found that vitamin K_3 or a crude lipid extract from *E. coli* satisfies this requirement. It is, however, unlikely that vitamin K_3 is the natural factor, since this compound has never been found in nature. Vitamin K_2 (*16*) and ubiquinone$_8$ (*17*) which occur in *E. coli* cells appear to be more likely candidates for the natural factor and attempts to identify the physiologically active substance are now in progress in this laboratory. It is interesting in this connection that ubiquinones have been implicated as a factor required for succinate and DPNH dehydrogenase systems of mammalian mitochondria (*18*). More recently, ubiquinone$_8$ and a lipoprotein have been suggested to be involved in the succinate-cytochrome *c* reductase system from *Azotobacter* (*19*) and the pyruvate-cytochrome b_1 reaction in *E. coli* (*20*), respectively.

The inability of 2, 6-dichlorophenol indophenol to be reduced by formate in the purified preparation also appears to be related to the loss of the factor just discussed. It is possible *in vivo* that this dye, unlike the

* Dr. K. Hori, personal communication.

other acceptors capable of directly reacting with formate dehydrogenase, is reduced by formate only through the lost factor or cytochrome b_1. Lester and Smith (*21*) have also reported that in beef-heart mitochondria the indophenol dye can not be reduced directly by succinate dehydrogenase, but is reducible under the mediation of cytochrome *b* or ubiquinone$_{10}$.

Earlier experiments by Gale (*1*) have shown that the oxygen uptake by a crude extract from *E. coli* in the presence of formate and a dye becomes inhibited at higher oxygen tensions. Similar observations have also been reported by Gest (*22*). In the present paper it was clearly demonstrated that the activity of solubilized formate dehydrogenase is reversibly inhibited by oxygen. This inhibition is quite remarkable; half maximal inhibition being attained in the presence of as low as 5% oxygen in the atmosphere. It may be probable that such high sensitivity of formate dehydrogenase to oxygen is connected to its functions in anaerobic metabolism such as nitrate respiration. Although the mechanism of this inhibition is not yet clear, it does not seem likely that oxygen competes with redox dyes as hydrogen acceptor, since no oxygen uptake was observed even at a low oxygen tension when formate alone was added to the purified preparation.

In summary, the results reported in this paper seem to support the scheme shown below for the electron-transport pathway from formate to nitrate in the particulate fraction of *E. coli* cells.

In this scheme, nitrate reductase is placed immediately after cytochrome b_1, since it has recently been confirmed that there is a direct interaction between these two components in the solubilized system (*23*).

SUMMARY

A preparation containing formate dehydrogenase, cytochrome b_1 and nitrate reductase was solubilized and partially purified from the particulate fraction of *E. coli* cells grown anaerobically under the conditions favorable for nitrate respiration. Some properties of formate dehydrogenase and cytochrome b_1 in this purified preparation were investigated. It was suggested that formate dehydrogenase is a metalloflavoprotein with essential sulfhydryl groups. Its activity was strongly but reversibly inhibited by molecular oxygen. The prosthetic group of cytochrome b_1 was decisively identified as protoheme, and it was found that the cytochrome is autoxidizable. The reduction of cytochrome b_1 by formate was shown to require, in addition to formate dehydrogenase, a lipid-soluble factor which could be replaced by vitamin K_3 in the solubilized system.

We are indefted to Drs. H. Maeno and S. Sakakibara for generous gifts of crude snake venom and crystalline protohemin, respectively.

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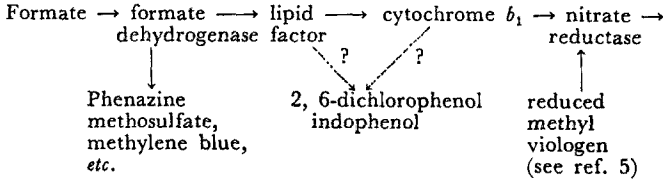

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