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FRAGMENTATION OF THE ELECTRON TRANSPORT CHAIN OF *ESCHERICHIA COLI*

PREPARATION OF A SOLUBLE FORMATE DEHYDROGENASE–CYTOCHROME b_1 COMPLEX

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SUMMARY

The electron transport system of *Escherichia coli* has been studied with particular reference to the particulate enzymes formate dehydrogenase (formate: cytochrome b_1 oxidoreductase, EC 1.2.2.1) and cytochrome b_1. A soluble preparation of a formate dehydrogenase–cytochrome b_1 complex was obtained by extraction of suitably grown cells with deoxycholate and high concentrations of $(NH_4)_2SO_4$. The dehydrogenase, a flavoprotein requiring sulphydryl groups for activity, is quantitatively extracted with a 20-fold purification and is largely free of other particulate enzymes.

A representative preparation contains about 0.5 mμmole flavin per mg protein, 1.5 mμmoles cytochrome b_1 per mg protein, 3% lipid and it oxidizes formate with ferricyanide as acceptor at a rate of 17 μmoles/min per mg protein.

The cytochrome b_1 of the preparation is readily reducible by formate. The functional linkage between the dehydrogenase and cytochrome component is lost on mild aeration or aging of the preparations. The communication between the two components can be restored by the addition of vitamin K_3. However, neither vitamin K nor ubiquinone could be detected in fresh active preparations and it is therefore unlikely that a quinone is involved as a natural intermediate in this portion of the electron transport system of *Escherichia coli*.

INTRODUCTION

In recent years the fragmentation and study of the electron transport system of animal cells have been the subject of intense study, and most of the individual components have now been recognised and isolated. However, comparatively little work has been carried out on the microbial electron transport systems, which vary considerably from one organism to another in number and type of cytochromes. On the one hand, anaerobic Clostridia contain no cytochromes, while other microorganisms such as yeast and *Bacillus subtilis* have the same number and type of cytochromes as mammalian cells, namely cytochromes a–a_3, b, c, and c_1 (ref. 1). *E. coli*, of particular interest in this present study, contains three cytochrome components, cytochromes

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b_1, a_1 and a_2 (ref. 1). Although *E. coli* cytochromes differ in number and type from those of mammalian systems, the fundamental organization appears to be similar. The cytochromes and primary dehydrogenases of the electron transport system, such as succinate dehydrogenase (EC 1.3.99.1) and $NADH_2$ dehydrogenase (EC 1.6.99.1) and also formate dehydrogenase (EC 1.2.2.1), are associated in the insoluble lipoprotein matrix of the cell's cytoplasmic membrane, as are the animal equivalents in the mitochondrion. Another dimension of the *E. coli* system reflecting the organism's ability to grow anaerobically, is the presence of nitrate reductase (EC 1.9.6.1) as part of this enzyme complex. This enzyme, linked through cytochrome b_1 to the primary dehydrogenases, is believed to provide a mechanism whereby substrates can be conveniently oxidized by the organism under anaerobic conditions, using nitrate as the terminal electron acceptor in place of oxygen[2].

Although the overall pattern of electron transport in *E. coli* is well recognised, the individual components with the exception of nitrate reductase[3] have not been isolated free of other enzymes, nor has their mechanism of interaction been elucidated. This communication describes the isolation and some of the properties of a soluble fragment of the electron transport system, a formate dehydrogenase–cytochrome b_1 complex. A brief account of the work has appeared earlier[4]. More recently ITAGAKI *et al.*[5,6] have independently described a closely related complex. Their preparation differs from that described here in that the formate dehydrogenase and cytochrome b_1 are not directly linked and their preparation contains high concentrations of nitrate reductase.

EXPERIMENTAL

Growth of the organism

E. coli B cells were cultivated aerobically in continuous culture at 30° on a medium made up of beef extract (0.3%), peptone (0.5%), NaCl (0.5%), glucose (1.0%), KH_2PO_4 (0.5%) adjusted to an initial pH of 6.5.

Disintegration of cells

Harvested, water-washed cells were suspended in 0.03 M potassium phosphate buffer (pH 6.8) to a final concentration of 40–60 mg protein per ml. The cell suspension, in 100-ml portions, was quantitatively disrupted at 0° in a specially designed blendor[7] by high speed agitation for 7 min with 130 ml of glass beads (diameter 0.10–0.18 mm). Glass beads and large cell fragments were then removed from the homogenate by centrifugation at 5000 × *g* for 20 min and the supernatant suspension separated. The small cell-particle fragments were then sedimented by centrifugation of the supernatant fluid at 55 000 × *g* for 30 min, collected, washed and resuspended in 0.01 M KCl solution. The small-cell-particle fraction hereafter denoted as the "cell residue" was the starting material for the enzyme isolation (*cf.* Fig. 1).

Analytical methods

Protein was determined by a modification of the biuret method[8]. The procedure of GREEN *et al.*[9] was used for flavin estimation. Cytochrome b_1 was calculated from the difference spectrum using the extinction coefficient for the α band of mammalian

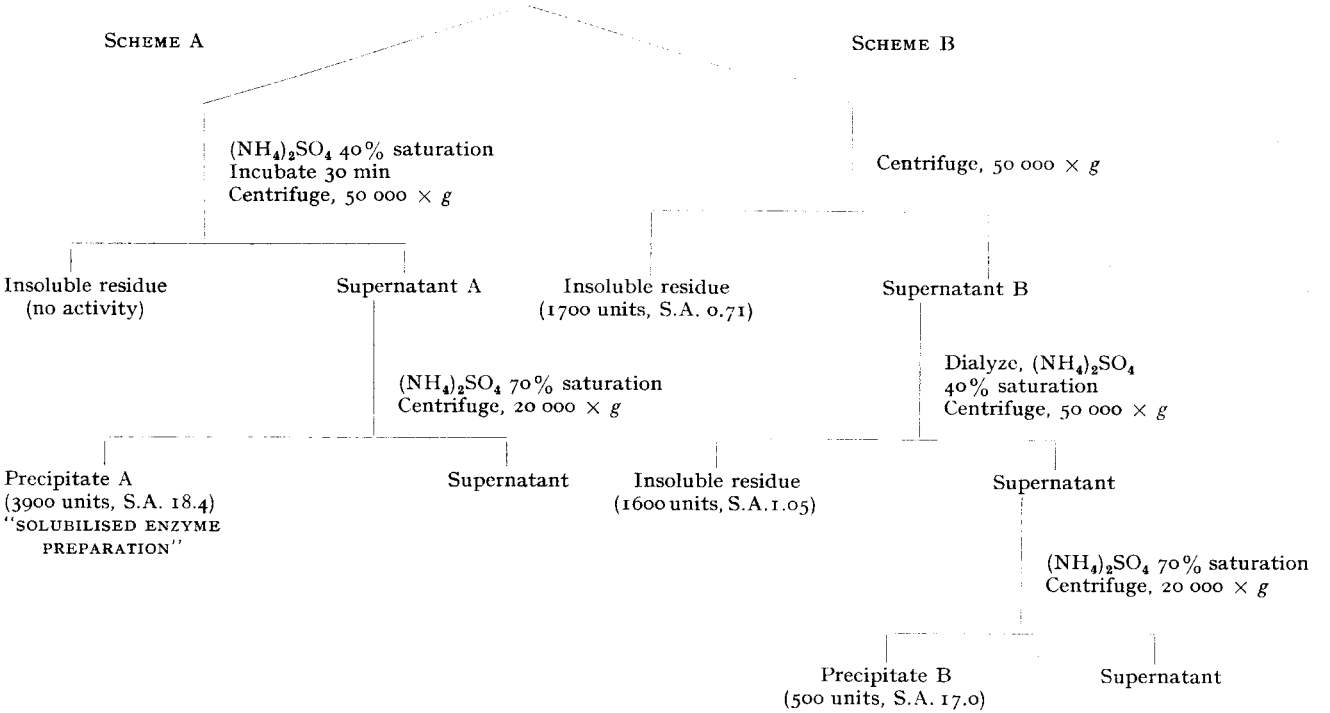

Fig. 1. Extraction procedures used for isolation of the solubilised formate dehydrogenase complex. Scheme A is the procedure routinely used. The total and specific formate dehydrogenase activities of the fractions are shown in brackets, with specific activity (S.A.) expressed as μmoles formate oxidized per min per mg protein and units as μmoles formate oxidized per min; one unit representing the amount of enzyme required to oxidise 1 μmole of formate per min.

cytochrome b[10] or as the pyridine haemochromogen by the method of BASFORD *et al.*[11]. There was good agreement in results obtained by these two methods of cytochrome b_1 estimation.

Ubiquinone and vitamin K were extracted from the heat dried samples with *iso*-octane and separated by chromatography on a column of Decalso resin as described by LESTER AND CRANE[12]. The separated coenzyme Q was estimated according to CRANE *et al.*[13] and vitamin K by the method of EWING *et al.*[14]. RNA was assayed as pentose according to the procedure described by DRURY[15]. Deoxycholic acid was determined as described by MOSBACH *et al.*[16].

Lipid was extracted with chloroform–methanol (1:1, v/v) at 40° and estimated gravimetrically as that fraction soluble in chloroform.

Enzyme assays

All assays of enzyme activity were performed at 30°.

Formate and succinate oxidase activities (formate:O_2 oxidoreductase and succinate:O_2 oxidoreductase) were determined by conventional manometric techniques. The formate system was assayed at pH 6.0 and the succinoxidase at pH 7.4. Each manometer contained 50 μmoles potassium phosphate buffer (pH 6.0 or 7.4), 5 mg bovine serum albumin, 5 μmoles $MgSO_4$ and 40 μmoles substrate with 20% KOH in the centre wells.

Formate dehydrogenase activity was assayed manometrically using ferricyanide as electron acceptor and measuring the rate of acid production in bicarbonate buffer as evolved CO_2. The manometer flasks contained in the main compartment 42 μmoles $NaHCO_3$, 5 mg bovine serum albumin, 5 μmoles $MgSO_4$ and enzyme preparation; in the side arm were 6 μmoles $NaHCO_3$, 40 μmoles sodium formate and 20–200 μmoles $K_3Fe(CN)_6$; the final reaction volumes were made to 3.2 ml. The flasks and manometers were equilibrated with 100% CO_2 so that the final pH was 6.0. Enzyme activity was markedly influenced by ferricyanide concentration and hence the double reciprocal plot method of SINGER *et al.*[17] was used to calculate activity at infinite ferricyanide concentration. Over the concentration range 7–70 mM ferricyanide a 3-fold increase in activity was observed. The activity determined at infinite concentration of acceptor was about six times greater than at 7 mM.

Succinate dehydrogenase was assayed manometrically with 100 μmoles ferricyanide as electron acceptor in a final concentration of 0.24 M $NaHCO_3$ in equilibrium with a gas mixture of 95% N_2 and 5% CO_2 to give a final pH of 7.4. The activity of this enzyme was not influenced by the concentration of ferricyanide and its activity was determined directly from CO_2 evolution.

$NADH_2$ oxidase ($NADH_2$:O_2 oxidoreductase) activity was calculated from the rate of decrease of absorbancy at 340 mμ of a solution containing 60 μmoles potassium phosphate (pH 7.5), 0.05 μmole EDTA, 3 mg bovine serum albumin and enzyme in a volume of 1.0 ml. $NADH_2$ dehydrogenase was assayed similarly to $NADH_2$ oxidase except that 2 μmoles $K_3Fe(CN)_6$ were added as an artificial electron acceptor. In estimating $NADH_2$ dehydrogenase in the cell residue, oxidase activity was inhibited by the further addition of 1 μmole KCN.

Nitrate reductase activity was determined as the amount of nitrite formed from nitrate by the enzyme within a 5-min period. Nitrite was estimated by the

method of SNELL *et al.*[18]. The assay was carried out anaerobically in Thunberg tubes with reduced benzyl viologen as the source of reducing equivalents for the enzyme system. The barrel of the Thunberg tube contained 80 μmoles potassium phosphate (pH 6.8), 5 μmoles $MgSO_4$, 1.5 mg freshly dissolved $Na_2S_2O_4$ and 10 μmoles KNO_3 in a total volume of 1.4 ml. To the side-arm was added 5 mg bovine serum albumin, 0.2 mg oxidized benzyl viologen and enzyme in a volume of 0.6 ml. The reaction was stopped by plunging the tubes into boiling water and exposing the contents to air. The coupled formate–nitrate reductase system was similarly assayed, except that 40 μmoles sodium formate in the main compartment replaced the $Na_2S_2O_4$ and the benzyl viologen was omitted.

RESULTS AND DISCUSSION

Conditions for growth of E. coli

The level of formate oxidase in aerobically grown *E. coli* was influenced by the growth conditions adopted for the organism. In particular variation of the initial pH of the glucose-nutrient broth medium affected both the cell yield and the formate oxidase activity of the cells. The maximal yield of cellular protein was obtained by growing the cells at an initial pH of 8.0 but maximal formate oxidase specific activity resulted from growth at pH 5.0. The highest yield of formate oxidase was obtained from cells grown around pH 6.5, it fell off sharply above and below this value. In addition to physical factors the composition of the growth medium also influenced the yield of the formate system. Recently ITAGAKI *et al.*[6] have reported that the yield of formate dehydrogenase from anaerobically grown cells was greatly increased by the inclusion of formate in a peptone–yeast extract medium. However in continuous culture under aerobic conditions in the present study the addition of formate to the nutrient broth medium resulted in a lower yield of formate oxidase from the cells.

Aerobic growth of the organism on simpler defined glucose or glycerol salts media was equally as prolific as that on nutrient broth but formate oxidase activity was much lower. The response of the organism to the different conditions of cultivation is clearly a complicated one and no ready explanation is available.

Solubilization and purification of the formate dehydrogenase–cytochrome b_1 complex

The starting material for the preparation of the formate dehydrogenase–cytochrome b_1 complex was the cell residue fraction, presumably largely a mixture of cell wall and cytoplasmic membrane fractions. The enzyme complex is very firmly bound to the cell residue and a number of procedures in common use for the solubilization of enzymes were initially investigated without success. These included digestion with phospholipase (EC 3.1.1.4) from venom of rattle snake *(Crotalus adamanteus)*, aqueous *n*-butanol extraction, acetone drying and 10% aqueous ethanol extraction at temperatures of 30–50°. In all of these instances either no significant extraction was achieved or the dehydrogenase was inactivated. The successful solubilization of the enzyme with the crude venom of *Trimeresurus flavoviridis* has been reported[6], presumably the result of a combination of enzymes present in crude venom as opposed to partially purified phospholipase enzyme.

Quantitative extraction of the formate dehydrogenase in a soluble form together with a considerable portion of the cell-residue cytochrome b_1 was achieved using a combination of sodium deoxycholate and high concentrations of $(NH_4)_2SO_4$ as set out in Scheme A of Fig. 1. The combined action of these two reagents was essential for the quantitative solubilization of the formate dehydrogenase. In the absence of $(NH_4)_2SO_4$ only about 60% of the total activity was extracted by the deoxycholate into the first supernatant (Fig. 1 Scheme B) and the bulk of this material is not in true solution. If the deoxycholate concentration was reduced by overnight dialysis and the extract then fractionated with $(NH_4)_2SO_4$, the bulk of the activity precipitated below 40% $(NH_4)_2SO_4$ saturation. This material was particulate in nature, it failed to dissolve on resuspension and dialysis. A small amount of activity was recovered precipitating between 40–70% $(NH_4)_2SO_4$ saturation; this fraction was similar in properties to the solubilized preparation obtained by the Scheme A procedure.

Precipitate A (Fig. 1) after resuspension in 0.01 M KCl–0.1 M sodium formate was dialyzed anaerobically overnight against the same salt solution and any sediment removed by centrifugation. The resultant red solution was the solubilized enzyme preparation used in the present studies. The enzyme activity remained in solution after 5 days dialysis against dilute salt solutions which reduced the residual deoxycholate from about 30 to 2–3 μg per mg protein. Centrifugation of the dialyzed material at 174 000 $\times$ g for 60 min did not sediment any active material and concentrations of $(NH_4)_2SO_4$ above 45% saturation were required to precipitate the complex which readily redissolved in dilute salt solutions. The preparation was therefore adjudged soluble.

The inclusion of formate in the procedure was found to be essential for the preservation of the functional linkage between the formate dehydrogenase and the cytochrome b_1 and this activity was retained for several weeks if the preparation was stored anaerobically in the presence of formate at 2°. Where necessary the formate and enzyme preparation could be conveniently separated by the use of Sephadex G25 columns. It was necessary to handle the preparations under anaerobic conditions as far as this was practical. The conditions of storage did not affect the dehydrogenase activity which was very stable.

Attempts at further purification of the solubilized enzyme preparation have not been successful but they gave some information on the nature of the material. In all fractionation procedures investigated, including the use of $(NH_4)_2SO_4$, solvents, $Ca_3(PO_4)_2$ gel, chromatography on DEAE-cellulose, starch gel and paper electrophoresis, the formate dehydrogenase–cytochrome b_1 complex did not behave as a single entity but spread over a wide range of fractions. None of the subfractions obtained showed significant purification of the dehydrogenase over the starting material and no separation of the dehydrogenase from the cytochrome b_1 was observed. The enzymes are thus bound in a physical as well as functional complex.

Examination of a variety of Precipitate A fractions and sub-fractions therefrom in the analytical ultracentrifuge showed that all were made up of four bands. However although the percentage of material in each peak showed some variation from one fraction to another the ultracentrifugal patterns were in the main similar. The fastest moving peak accounted for over half the material while the three smaller peaks were each of the same order of magnitude. The constancy of the ultracentrifuge pattern and the lack of purification of the enzyme irrespective of the particular

sub-fraction used for analysis suggest that the material readily associates or polymerizes to macromolecular complexes in such a way as to yield the same ultracentrifuge pattern. The spontaneous aggregation of cytochrome components isolated from animal tissues has now been described on several occasions[19,20].

Enzymic properties of solubilized preparation

The enzymic activities of the cell residue and solubilized preparation are compared in Table I. Formate dehydrogenase was purified about 20-fold by the procedure adopted and a 100% recovery of enzyme was obtained. The specific activity of the preparation was about 17 μmoles formate oxidized per min per mg protein, which is some 8-fold higher than that recently reported by ITAGAKI *et al.*[6]. Formate oxidase activity was only about 1% of the total activity present in the cell residue and this residual activity is probably due to the slow auto-oxidation of the flavin and cytochrome b_1.

TABLE I

ENZYME ACTIVITIES OF CELL RESIDUE AND FORMATE DEHYDROGENASE–CYTOCHROME b_1 COMPLEX

The solubilized enzyme complex (240 mg protein) was prepared from 4750 mg of cell-residue protein. Specific activity is expressed as μmoles substrate reacted per min per mg protein. Total activity is the product of specific activity and total protein of the fractions.

Enzyme	*Cell residue*		*Formate dehydrogenase–cytochrome b_1 complex*	
	Specific activity	*Total activity*	*Specific activity*	*Total activity*
Formate oxidase	1.0	4750	0.20	48
Formate dehydrogenase	0.89	4228	17.7	4248
Succinate oxidase	0.08	380	0	—
Succinate dehydrogenase	0.11	523	0	—
$NADH_2$ oxidase	0.15	714	0	—
$NADH_2$ dehydrogenase	0.21	998	0.54	130
Nitrate reductase	0.25	1188	0.11	26
Formate–nitrate reductase*	0.12	570	0	—

* Formate:nitrate oxidoreductase.

The dehydrogenase evidently requires sulphydryl groups for its activity as shown by its complete inhibition by 0.1 mM *p*-chloromercuribenzoate and a 70% inhibition by 1 mM iodoacetic acid. 5 mM cyanide also inhibits the dehydrogenase by about 60% which may suggest a possible metal ion involvement.

The enzymes succinate dehydrogenase and oxidase and $NADH_2$ oxidase although present in the cell residue were not extracted into the soluble fraction but some 13% of the $NADH_2$ dehydrogenase activity was extracted. It is of interest to note that the synthesis of the formate oxidase system is markedly favoured by the cells over the succinate and $NADH_2$ oxidase systems under the conditions of growth described herein as judged by the specific activities recorded in Table I. With reduced benzyl viologen as electron donor only trace amounts of nitrate reductase were detected in the soluble complex while there was no activity with formate as electron donor.

Spectroscopic data

Direct spectra of the solubilized preparation are shown in Fig. 2. The oxidized preparation has a Soret peak at 412 mμ and a very small second peak at 523 mμ, after reduction with hyposulphite peaks appear at 559, 530 and 428 mμ. The positions of the absorption bands correspond closely with those recorded in the literature for cytochrome b_1 (ref. 1, 6, 21). A trough at about 460 mμ indicated the presence of flavin which was confirmed by direct analysis (Table II). No traces of cytochrome a_1 or a_2 were ever found in these preparations.

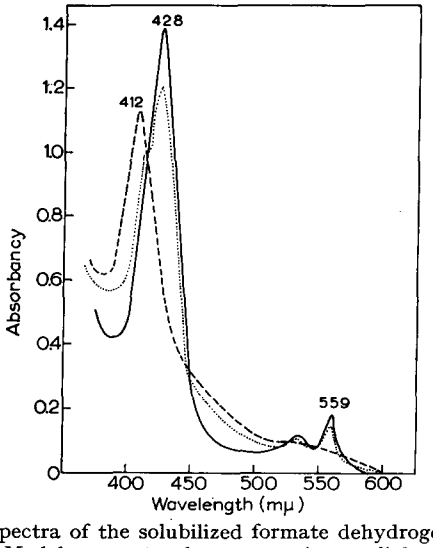

Fig. 2. Direct absorption spectra of the solubilized formate dehydrogenase–cytochrome b_1 complex, recorded with a Cary Model 14 spectrophotometer. A 4-cm light path-cell contained 0.9 mg enzyme protein per ml. — — —, oxidized spectrum; . . . . . , preparation reduced with formate; ———, hyposulphite-reduced preparation. $A_{428\ m\mu}/A_{559\ m\mu} = 7.7$; $A_{428\ m\mu}/A_{412\ m\mu} = 1.26$.

Pyridine haemochromogen prepared and reduced as described by BASFORD *et al.*[11] showed a direct absorption spectrum with maxima at 557, 523 and 419 mμ. The positions and relative heights of the absorption peaks corresponded closely to the values of protohaem pyridine haemochromogen reported by MORTON[21]. The extinction coefficient for 559 to 575 mμ (reduced) was calculated from the value for pyridine ferroprotoporphyrin obtained by PAUL *et al.*[22], to be 22.6 $mM^{-1}\,cm^{-1}$ assuming one protohaem per mole. From this figure other extinction coefficients can be calculated from the spectral properties of the dehydrogenase complex.

Most of the cytochrome b_1 of the complex was reducible by formate although the extent of the reduction varied among the preparations from about 40–100% (Fig. 2). This variation is presumed due to the preparation's lability in the presence of O_2. The enzyme rapidly loses the ability to reduce cytochrome b_1 when stored or handled extensively in air. Although care was taken to manipulate the preparation as far as possible in the absence of air it is not practical to exclude it at all stages.

There was no functional linkage of $NADH_2$ dehydrogenase or of nitrate reductase to the cytochrome b_1. The cytochrome was not reduced in the presence of $NADH_2$ nor was reduced cytochrome b_1 reoxidized by the addition of nitrate.

TABLE II

COMPOSITION OF FORMATE DEHYDROGENASE–CYTOCHROME b_1 COMPLEX AND CELL RESIDUE

The values given are typical of a routine preparation of the complex. The specific activity of this preparation was 17.6 μmoles formate oxidized per min per mg protein.

Component	*Cell residue*	*Formate dehydrogenase–cytochrome b_1 complex*
	Percentage	
Protein	66	95
Lipid	20	3.4
RNA	—	2.7
	mμmoles per mg protein	
Cytochrome b_1	0.19	1.54
Flavin (total)	0.18	0.54
Flavin (acid-extractable)	0.15	0.39
Ubiquinone	0.52	0
Vitamin K_1	0.47	0

Composition

The composition of representative cell residue and soluble fractions are shown in Table II. The soluble complex is largely made up of protein, together with a small amount of lipid (3%) and RNA (3%). Earlier we reported the presence of up to 30% low molecular weight RNA in the soluble preparation[4] but over a 5-day dialysis period the amount of RNA is greatly reduced. The RNA has no functional significance and can be completely removed by RNAase digestion followed by dialysis without effect on the enzymic activity of the preparation.

The cytochrome b_1 levels in the complex ranged from 0.7–2.5 mμmoles per mg protein (Table II). The different cytochrome content in the complex to some extent reflects the variable levels in the cell residue which ranged from 0.15–0.24 mμmole per mg protein with an average value around 0.19 mμmole per mg protein (Table II). The enzyme complex is clearly a fragment of the basic electron transport chain made up of the total formate dehydrogenase content of the organism and a variable proportion of the total cytochrome b_1. The complex is thus unlike the yeast lactate dehydrogenase–cytochrome b_2 system which is a complete unit of constant stoichiometry and not part of the main electron transport chain[21]. The formate system is on the other hand similar to several described succinate dehydrogenase–cytochrome complexes isolated from beef heart[23] and bacteria[24].

Examination of the absorption spectra obtained with formate and hyposulphite as reductants showed that only part of the flavin was reduced by formate (Fig. 2.) The total flavin was made up of an acid-extractable component and a smaller amount released only after proteolytic digestion (Table II). The nature of the flavin of the formate dehydrogenase is not certain, but a variety of experiments suggest it is of the acid-extractable type.

Nature of the dehydrogenase–cytochrome linkage

ITAGAKI *et al.*[5,6] have recently described a soluble formate dehydrogenase–cytochrome b_1 preparation in which the two components were not functionally linked. In the presence of vitamin K_3 (menadione) or a crude lipid fraction from *E. coli* cytochrome b_1 was reduced by formate. These observations have led the authors to suggest that vitamin K may function as an intermediate carrier in the organism between the dehydrogenase and cytochrome b_1.

The present experiments do not however support the conclusions of ITAGAKI *et al.*[6]. Although significant amounts of vitamin K were found in the cell residue none was detected in the soluble complex in which cytochrome b_1 was rapidly reduced by formate (Table II). The vitamin K component of the cell residue was identified on purification as K_1 by its characteristic absorption maxima at 243, 249, 260 and 269 mμ[14].

As prepared herein the cytochrome b_1 of the complex was immediately reduced by formate but the preparations lose this property if aged in air in the absence of substrate, briefly for 2–3 h at 30°, or over several days at 0°. The addition of menadione restores the capacity of such aged preparations to reduce cytochrome b_1 with formate. The rate of reduction is slow, taking 3–4 min which is of the same order as that reported by ITAGAKI *et al.*[6]. The slow rate of this reaction and the absence of vitamin K from the soluble preparation make it unlikely that a vitamin K derivative mediates between the two electron transport components in their native state. Menadione presumably functions as an artificial intermediate which can be alternately reduced and oxidized by the two enzymes.

It appears also that the benzoquinone ubiquinone is not a component of the formate dehydrogenase–cytochrome b_1 system. It occurs in the cell residue in high concentration but was not detected in the soluble fraction (Table II). Also UQ_6, UQ_8 or UQ_{10} when added to the aged preparations, unlike vitamin K_3, failed to function as carriers between the dehydrogenase and the cytochrome. These findings are consistent with observations on the analogous succinate dehydrogenase–cytochrome *b* complex prepared from beef heart mitochondria[23]. In the animal system ubiquinone mediates between the primary dehydrogenase and cytochrome c_1 while cytochrome *b* is reduced directly by the dehydrogenase[23,24].

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