Biochimica et Biophysica Acta, 591 (1980) 275—288

BBA 47870

ISOLATION AND FUNCTIONAL ASPECTS OF THE FUMARATE REDUCTASE INVOLVED IN THE PHOSPHORYLATIVE ELECTRON TRANSPORT OF *VIBRIO SUCCINOGENES*

GOTTFRIED UNDEN, HEINZ HACKENBERG and ACHIM KRÖGER

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, 8000 München 2 (F.R.G.)

(Received August 3rd, 1979)

(Key words: Electron transport; Flavoprotein; Fumarate reductase; Phosphorylation; (Vibrio succinogenes)

Summary

1. The fumarate reductase of *Vibrio succinogenes*, the terminal component of the electron transport chain of the bacterium, was extracted from the cytoplasmic membrane with Triton X-100 and purified to homogeneity (approx. 30-fold) by means of chromatography on hydroxyapatite and DEAE-Sephadex. The enzyme eluted from the ion-exchange column in two forms, one containing and the other lacking cytochrome *b*.

2. The enzyme lacking cytochrome *b* consisted of two peptides (M_r 79 000 and 31 000) which were present in a molar ratio of 1 : 1. The cytochrome-containing species contained an additional peptide (M_r 25 000) which was present in twice the molar amount of the others (2 : 1 : 1).

3. The hydrodynamic properties indicate that the functional enzymes consist of only one set of the corresponding peptides.

4. Each of the two enzyme molecules contains one protein-bound FAD which is linked to the M_r 79 000 peptide. Both enzyme species contain 9—10 mol iron-sulfur per mol of FAD which is associated with the M_r 79 000 and the M_r 31 000 peptide. Cytochrome *b* is present in an amount of 2 mol/mol of FAD, half of the cytochrome *b* has a midpoint potential of —20 mV.

5. The enzyme catalyzed two types of reaction. (a) Fumarate reduction by viologen radicals or anthrahydroquinonesulfonate, as well as succinate oxidation by ferricyanide or methylene blue, was independent of cytochrome *b*. (b) The activities of fumarate reduction by naphthohydroquinones and those of succinate oxidation by naphthoquinones were cytochrome *b*-dependent. This indicates that the electron transport from menaquinone to fumarate

---

Trivial names: menadione, 2-methyl-1,4-naphthoquinone; menadiol, 2-methyl-1,4-naphthohydroquinone.

reductase in the membrane of the bacterium is mediated by a single component, cytochrome *b* (−20 mV).

6. The K_m for fumarate was 0.35 mM with menadiol as the donor and that for succinate was 20 mM in the reverse reaction. Succinate oxidation was competitively inhibited by fumarate with a K_i of 0.35 mM.

Introduction

Fumarate reduction by various hydrogen donor substrates is catalyzed by the cytoplasmic membranes of a great variety of bacteria which are capable of growing under anaerobic conditions [1—3]. These reactions are catalyzed by electron transport chains which contain a menaquinone and were shown to be associated with electron transport phosphorylation in many cases [1—4]. Although it is generally assumed that a fumarate reductase is the terminal electron transport component in these chains, the enzyme has not yet been isolated and characterized. An earlier preparation of the enzyme from *Propionibacterium pentosaceum* was non-homogenous and its composition was not investigated in detail [5]. Soluble fumarate reductases were isolated from yeast [6] and from *Veillonella alcalescens* (formerly *Micrococcus lactilyticus*) [7] and found to be flavoproteins. The metabolic function of these fumarate reductases is probably different from that of the membrane-bound enzymes.

Vibrio succinogenes can generate the ATP required for growth from the reduction of fumarate by either formate or hydrogen [1,3,4,8—11]. This is accomplished by a system of electron transport phosphorylation which is especially suited for studying the mechanism of energy transduction [3,4,9—13]. The electron transport from formate or hydrogen to fumarate is associated with the generation of an electrochemical proton potential across the membrane [1,3,4,10,11] which possibly acts as the coupling device between electron transport and phosphorylation [1,2,4,10,14]. An understanding of the mechanism of generation of the proton potential requires that the composition and properties of the fumarate reductase are known [10].

This paper reports the isolation and properties of the fumarate reductase of *V. succinogenes* as well as the sequence of the electron transport components between menaquinone and fumarate reductase. The structure of the flavin prosthetic group of this enzyme has been given in a previous publication [15]. The solubilization and purification of the enzyme follows essentially the procedures which have been developed to isolate membrane proteins, especially the ADP, ATP carrier of mitochondria [16—20]. A part of the results of this paper was published in an earlier review [4].

Methods

Purification of fumarate reductase

V. succinogenes [8] was grown and stored as described previously [12,20].

Step 1. The cells were lysed and extracted with Tween 80 and Triton X-100 as described [20]. However, 5 mM succinate was present in the buffers and NaN_3 was omitted. Dithiothreitol (2 mM) was present in the buffers containing Tween or Triton.

Step 2. The Triton extract (300 ml containing approx. 200 mg protein) was applied to a hydroxyapatite [21] column (100 ml) as described [20]. However, the column was equilibrated and rinsed with a buffer containing 10 mM instead of 30 mM phosphate, and 2 mM fumarate and 2 mM dithiothreitol in addition. The fractions containing the enzyme at a sufficiently high specific activity were pooled, concentrated approx. 4 times by pressure dialysis and dialysed for 16 h against an anaerobic buffer containing 0.05% Triton, 20 mM Tris, 5 mM succinate and 2 mM $Na_2S_2O_4$ at pH 7.5 and 0°C.

Step 3. The solution obtained from step 2 was subjected to chromatography on DEAE-Sephadex A-25 as described in the legend of Fig. 1.

Enzymic activity

The activities of fumarate reduction with the viologen radicals (methyl viologen or benzyl viologen) or with anthrahydroquinonesulfonate, as well as those of succinate oxidation by methylene blue or ferricyanide were measured under anaerobic conditions at 25°C as described previously [10,12].

Fumarate reduction by dimethylnaphthohydroquinone or menadiol, as well as succinate oxidation by dimethylnaphthoquinone or menadione, were measured with a dual-wavelength spectrophotometer. The wavelengths and the molar difference extinction coefficients (oxidized − reduced) used for dimethylnaphthoquinone and menadione were given previously [20]. The measurements were made in a 0.5 cm cuvette which contained 1 ml of 50 mM phosphate, pH 7.5, at 25°C and 0.2 mM of the quinone. The solutions were aspirated with N_2 for 2 min before the enzyme was added, and kept under an atmosphere of N_2. For measuring succinate oxidation, the reactions were started by the addition of 20 mM succinate. Fumarate reduction was recorded after reduction of the quinones by 0.5 mM KBH_4 followed by the addition of 1 mM fumarate.

The unit of activity with the various dyes represents the reduction of 1 μmol fumarate or the oxidation of 1 μmol succinate per min under the given conditions.

Protein determination

Protein was measured by counting the radioactivity which had been incorporated by growing the bacteria in the presence of [^{3}H]leucine (40 μCi/l). The specific activity of the protein was determined in the Triton extract (step 1) using the biuret method with KCN [22]. The extinction coefficient at 546 nm (0.266 $l \cdot g^{-1} \cdot cm^{-1}$) was measured with bovine serum albumin.

Other assays

Flavin [12], cytochromes *b* and *c* [13] and sulfide [20] were measured as described previously. The amount of Triton bound to fumarate reductase was determined by measuring the ^{3}H-labeled detergent which eluted together with the enzyme from a hydroxyapatite column [18]. Phospholipids were measured by phosphate analysis after hydrolysis [23]. Average molecular weight was assumed to be 800.

Results

Isolation of fumarate reductase

The procedures used for the extraction of fumarate recductase from the membrane of the bacterium and for purification are similar to those previously applied to the formate dehydrogenase of *V. succinogenes* [20]. The purification of the enzyme was followed by measuring the content of protein-bound FAD (Table I) which was shown to be the prosthetic group of fumarate reductase in previous work [12,15]. The specific activity of fumarate reduction by benzyl viologen of the preparations is linearly related to the content of bound FAD, except for the enzyme obtained by chromatography on hydroxyapatite. At this step the specific activity was only approx. 60% of that expected from the FAD content.

After lysis of the cells the enzyme was extracted from the membrane fraction with Triton X-100 (step 1). Thus, 52% of the bound FAD was solubilized and the content of FAD increased 5.6-fold. The solubilized enzyme was absorbed by hydroxyapatite and eluted as a single band at approx. 35 mM phosphate. The mixture of the fractions used for further purification contained 78% of the FAD and the purification was 3.1-fold.

After dialysis, the fumarate reductase obtained from step 2 was absorbed by a DEAE-Sephadex column (step 3). The enzyme eluted from this column on application of a linear NaCl gradient appeared in two bands (Fig. 1). The enzyme eluting at approx. 10 mM NaCl contained cytochrome *b*, whilst that eluting at approx. 100 mM NaCl did not. Cytochrome *b* was steadily eluted by the equilibration buffer before the NaCl gradient was applied. In other experiments the yield of fumarate reductase lacking cytochrome *b* increased with the volume of the equilibration buffer used for rinsing of the column, whilst that of the enzyme containing cytochrome *b* decreased. This indicates that the enzyme lacking cytochrome *b* is formed from fumarate reductase containing cytochrome *b* during chromatography. The content of bound FAD of the species containing cytochrome *b* was 1.1 times greater than in the preparation

TABLE I

PURIFICATION OF FUMARATE REDUCTASE

	Reduction of fumarate by benzyl viologen		Bound FAD		Cytochrome *b*	
	(units/mg protein)	(units)	(μmol/g protein)	(nmol)	(μmol/g protein)	(nmol)
Cell homogenate	6.6	11 300	0.32	540	0.51	875
Step 1						
Triton extract	26.8	4 280	1.8	282	5.0	1000
Step 2						
Chromatography on hydroxyapatite	47.5	2 000	5.5	220	10.1	400
Step 3						
Chromatography (a)	92	830	6.3	57	11.8	76
on DEAE-Sephadex (b)	140	760	9.1	49	<0.4	—

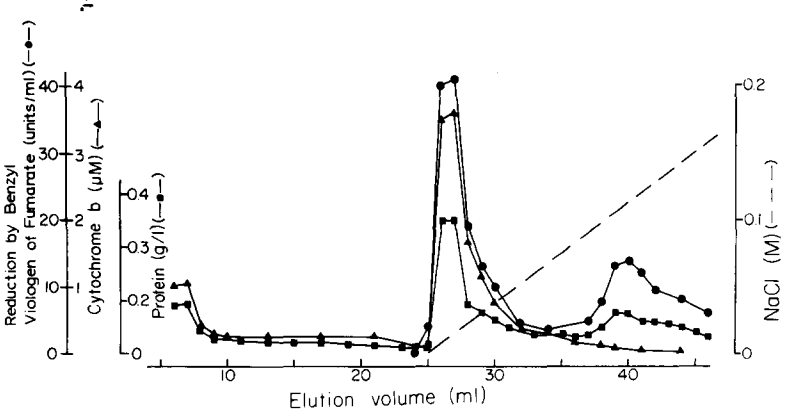


Fig. 1. Chromatography of fumarate reductase on DEAE-Sephadex (step 3). The preparation obtained from step 2 (20 ml containing 0.25 g protein/l) was applied to DEAE-Sephadex A-25 column (80 ml, 2 cm inner diameter) which had been equilibrated with an anaerobic buffer containing 0.05% Triton X-100, 20 mM Tris, 5 mM succinate and 5 mM dithiothreitol at pH 7.5 and 0°C. After rinsing with two column volumes of this buffer, the enzyme was eluted with a linear NaCl gradient (0—200 mM) in this buffer (320 ml) at a flow velocity of 80 ml/h.

obtained from step 2, and that of the enzyme lacking the cytochrome was 1.7 times greater. The overall yield of the purification procedure was 20% according to bound FAD. The overall purification was 20- and 28-fold for the enzyme species with and without cytochrome *b*, respectively. The content of cytochrome *b* was increased to approximately the same extent (23-fold) as that of FAD.

Homogeneity

The preparation from step 3 which contains cytochrome *b* was subjected to gel filtration as a test for the homogeneity of the enzyme (Fig. 2). A single symmetrical band of activity coincided with cytochrome *b* and the bulk of the protein. The specific activities and the content of cytochrome *b* were nearly equal in the main fractions of the band. The small shoulder of the protein band indicated the presence of protein of smaller molecular weight which amounted to approx. 10% as calculated from the activity of the corresponding fractions. A similar result was obtained from sucrose density gradient centrifugation of the preparation (not shown). These results suggest that the preparation was approx. 90% homogeneous. The preparation did not contain formate dehydrogenase, hydrogenase or nitrate reductase which were present in the membrane fraction.

Composition

The fumarate reductases containing and lacking cytochrome *b*, as obtained from step 3 of the preparation procedure, contained 6.0 or 8.8 μmol FAD/g protein, which were covalently bound (Table II). Both preparations contained 9—10 mol of non-heme iron and acid-labile sulfur per mol of FAD, indicating that iron-sulfur groups are constituents of the enzyme. Molybdenum, the prosthetic group of formate dehydrogenase, cytochrome *c* and acid-extractable FAD, although present in the membrane fraction of the bacterium, were absent from both preparations. The content of menaquinone (0.2—4 μmol/g

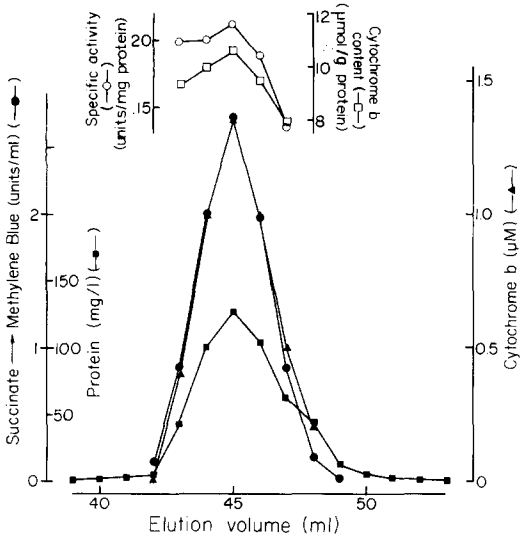


Fig. 2. Gel filtration of fumarate reductase. The enzyme containing cytochrome *b* as obtained from step 3 (0.2 ml containing 4.6 g protein/l) was applied to a Sepharose CL-6B column (1 cm inner diameter and 1 m length). A buffer containing 0.1% Triton X-100, 0.2 M NaCl and 10 mM morpholinopropanesulfonate at pH 7.2 and 0°C was used for equilibration and elution. The flow velocity was 1.8 ml/h. The recovery of protein was 56%.

protein) varied greatly from one preparation to the other, similar to that of the phospholipids (Table III). Because the reactions given in Table IV were catalyzed also by preparations which were virtually free of menaquinone, this compound is not regarded as an essential constituent of fumarate reductase.

The content of cytochrome *b* was approx. 2 mol/mol FAD (Table I). Approx. 40% of the cytochrome was reduced on the addition of succinate. Redox titration revealed that approximately equal amounts of cytochrome *b* (—200 mV) and cytochrome *b* (—20 mV) were present in the enzyme. The difference spectra (reduced — oxidized) of the cytochromes at the temperature of liquid N_2 are given in Fig. 3. With dimethylnaphthohydroquinone (E'_0 = —80 mV) only cytochrome *b* (—20 mV), which is characterized by a peak at 560 nm, is reduced (spectrum I). The spectrum (II) of cytochrome *b* (—200 mV) was recorded with dithionite, which reduces both cytochromes, in the

TABLE II

COMPOSITION OF FUMARATE REDUCTASE

Preparations of step 3 were analyzed. Non-heme iron was measured after extraction with trichloroacetic acid according to method C of Brumby and Massey [24].

	Cytochrome *b* present		Cytochrome *b* absent	
	(μmol/g protein)	(mol/mol bound FAD)	(μmol/g protein)	(mol/mol bound FAD)
Protein-bound FAD	6.0 ± 0.3	—	8.8 ± 0.3	—
Sulfide	53 ± 5	9.3	81 ± 10	9.6
Non-heme iron	56 ± 5	9.7	83 ± 10	9.8

TABLE III

HYDRODYNAMIC PROPERTIES OF FUMARATE REDUCTASE

The partial specific volumes of the complexes were calculated from the contents of Triton and phospholipid [27] using an average partial specific volume of protein of 0.725 ml/g [28]. The Svedberg equation was used for calculating the molecular weights of the complexes from the sedimentation coefficients and the Stokes radii. The molecular weight of the protein of the enzymes was calculated from those of the complexes and the contents of Triton and phospholipids [27]. The measurements were made with the enzymes in 0.05% Triton X-100, 0.1 M NaCl, 5 mM malonate and 20 mM Tris at pH 7.5. Analytical ultracentrifugation (Beckman, Model E) was performed at an enzyme concentration of 0.5 g protein/l using the absorbance of the cytochrome at 416 nm. Sedimentation equilibrium patterns at 5°C were recorded after 23 h at 4800 and 6200 rev./min and consistent results were obtained. The sedimentation coefficient was measured at 52 000 rev./min and 20°C. The sedimentation coefficient of the enzyme lacking cytochrome *b* was determined by sucrose density gradient centrifugation with 5—20% sucrose [28] in the buffer given above at 5°C. Rotor TV-865 (Du Pont Instruments) was used for centrifugation (100 min). Alcohol dehydrogenase from yeast (sedimentation coefficient 7.4 S) was used as the reference protein. The enzymes were detected by their enzymic activities. The Stokes radii were determined by gel filtration as described for the experiment of Fig. 2 [29]. Calibration was performed with seven proteins, the Stokes radii of which ranged between 4.1 and 7.3 nm [20]. Fumarate reductase was detected by its enzymic activity.

	Cytochrome *b*	
	Present	Absent
Triton content (g/g protein)	0.43	0.1
Phospholipid content (g/g protein)	0.2	0.11
Partial specific volume of the complex (ml/g)	0.80	0.76
M_r of complex (from sedimentation equilibrium)	326 000	—
M_r of protein (from sedimentation equilibrium)	200 000	—
Sedimentation coefficient (S)	10.5	7.15
Stokes radius (nm)	6.25	4.3
M_r of complex (from Stokes radius and sedimentation coefficient)	378 000	147 000
M_r of protein (from Stokes radius and sedimentation coefficient)	232 000	121 000

TABLE IV

ACTIVITIES OF FUMARATE REDUCTASE

The activities of dye oxidation and reduction were measured as described in Methods. Succinate respiration was measured with the enzyme in 50 mM phosphate at pH 7.4 and 25°C using a Clark-type oxygen electrode.

Reaction	E'_0 of dye (mV)	Activity with or without cytochrome *b* (units/mg protein)		K_m for dye (mM)
		Without	With	
Methyl viologen radical → fumarate	—446 [30]	255	226	—
Benzyl viologen radical → fumarate	—350 [30]	140	92	—
Anthrahydroquinone-2-sulfonate → fumarate	—225 [31]	7.1	3.5	—
Succinate → $Fe(CN)_6^{3-}$	360 [31]	0.53	0.76	—
Succinate → methylene blue	11 [31]	0.74	1.0	—
Succinate → O_2	—	—	0.56	—
2,3-Dimethyl-1,4-naphthohydroquinone → fumarate	—80 [31]	<0.01	7.2 *	0.12
Menadiol → fumarate	—1 [32]	<0.01	2.2 *	0.20
Succinate → 2,3-Dimethyl-1,4-naphthoquinone	—80	0	0.82	—
Succinate → menadione	—1	0	5.0 *	—

* *V* with respect to dye concentration.

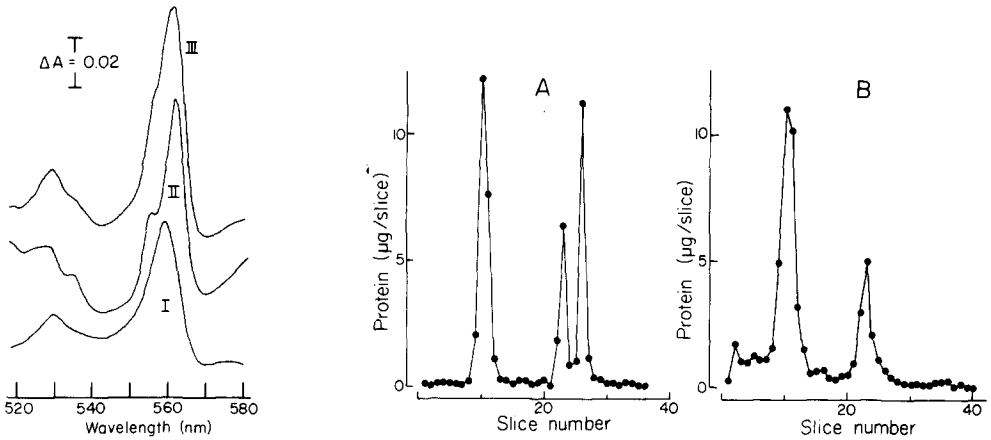


Fig. 3. Difference spectra at −196°C of fumarate reductase containing cytochrome *b*. The preparation of step 3 (0.3 g protein/l) in an anaerobic buffer containing 0.05% Triton X-100, 20 mM Tris, 0.5 M sucrose, at pH 7.5, was used. The optical path-length was 0.1 cm. The following additions were made. (I) Sample cuvette, 2 mM dimethylnaphthohydroquinone; reference cuvette, 2 mM ferricyanide. (II) Sample cuvette, dithionite; reference cuvette, 2 mM dimethylnaphthohydroquinone. (III) Sample cuvette, dithionite; reference cuvette, 2 mM ferricyanide.

Fig. 4. Polyacrylamide gel electrophoresis of fumarate reductase in the presence of sodium dodecylsulfate. Electrophoresis was performed according to Neville [25]. The gels (11% polyacrylamide) were 12 cm long and 0.5 cm in diameter. The enzyme (step 3) was lyophilized and the following solutions were added to a sample containing approx. 40 μg protein: 10 μl 0.5 M dithiothreitol and 40 μl of 5% sodium dodecyl sulfate in 40 mM borate and 40 mM Tris at pH 8.5. The mixture was kept at 40°C for 45 min before electrophoresis. (A) 47 μg of the enzyme containing cytochrome *b*, and (B) 60 μg of the preparation lacking cytochrome *b* were used. The gels were cut into 2-mm pieces which were digested for 3 h at 60°C with 0.5 ml NCS-solubilizer (Amersham/Searle, Arlington Heights, IL, U.S.A.). The solution was then diluted with 12 ml scintillator solution and analyzed for tritium. The specific radioactivity of the protein was 158 (A) and 392 dpm · μg^{-1} (B) and the recovery was greater than 90%.

sample cuvette, and dimethylnaphthohydroquinone in the reference cuvette. This spectrum shows a peak at 563 nm and a shoulder at 557 nm. The absorbance difference between fully reduced and fully oxidized fumarate reductase is represented by spectrum III.

Subunits of the enzyme

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that fumarate reductase containing cytochrome *b* (Fig. 4A) consists of three peptides of molecular weights 79 000, 31 000 and 25 000, as determined by interpolation from reference protein values. Gels of fumarate reductase without cytochrome *b* yielded only two peptides of molecular weights 79 000 and 31 000. The smallest peptide represents cytochrome *b*, since it is missing in the cytochrome-free preparation. The biggest peptide was fluorescent after incubation of the gel in 10% acetic acid. The fluorescence was visible on excitation with an ultraviolet lamp on gels loaded with only 5 μg of protein. The smaller peptides did not show fluorescence under these conditions on gels loaded with 30 μg of protein. The fluorescence excitation spectrum of the M_r 79 000 peptide in acetic acid showed two bands with peaks at approx. 345 and 445 nm and was thus similar to the absorption and excita-

tion spectra of 8α-histidylflavins [15,26]. Therefore, the M_r 79 000 peptide contains the FAD of fumarate reductase.

The relative molar amounts of the peptides were calculated from the radioactivity incorporated by growth of the bacteria in the presence of [^{3}H]leucine, on the basis that the leucine content is the same in each of the peptides. The cytochrome-free fumarate reductase contained 58% of the protein in the M_r 79 000 and 23% in the M_r 31 000 peptide. Division of the contents by the corresponding molecular weight showed that the two peptides are present in the molar ratio of 1 : 1. The distribution of the protein of the enzyme containing cytochrome *b* was 48, 19 and 28% in the M_r 79 000, 31 000 and 25 000 peptides, respectively; this gives molar ratios of 1 : 1 : 1.8. Thus, in both preparations the molar ratio of the two larger peptides is approx. 1 : 1. The ratio of the amounts of the M_r 79 000 and 25 000 peptides is consistent with that of the contents of FAD and cytochrome *b* (Table I).

To find out which of the peptides of fumarate reductase contains the iron-sulfur, the preparation containing cytochrome *b* was subjected to gel filtration in the presence of low concentrations of sodium dodecylsulfate (Fig. 5). Due to the denaturing action of sodium dodecylsulfate the recovery of sulfide was only 20%, whilst that of protein was greater than 90%. None of the fractions contained fumarate reductase activity with benzyl viologen radical as the donor. As established by gel electrophoresis of the protein of each fraction, the M_r 79 000 peptide was the first to elute from the column followed by the M_r 25 000 and 31 000 peptides. The proportions of the three peptides were evaluated from the photometric scans of the gels. From these proportions the molar concentrations of the peptides were calculated using the protein contents

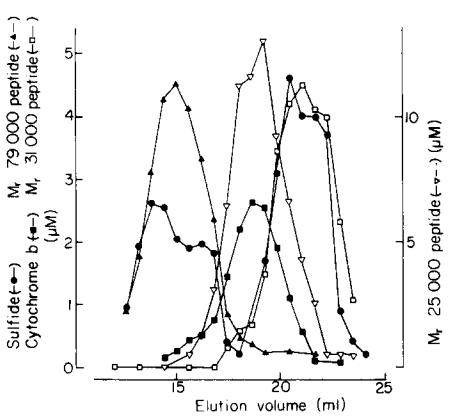


Fig. 5. Gel filtration of fumarate reductase in the presence of dodecyl sulfate. The enzyme containing cytochrome *b* (0.5 ml containing 7 g protein/l) in approx. 0.5% Triton X-100 and 0.5% sodium dodecyl sulfate was applied to a Sephacryl S-300 column (1 cm inner diameter and 1 m length). A buffer containing 0.05% Triton X-100, 0.015% sodium dodecyl sulfate, 20 mM Tris, 5 mM malonate, 1 mM dithiothreitol and 1 mM diisopropylfluorophosphate at pH 8.5 and 0°C was used for equilibration and elution. The flow velocity was 1.8 ml/h. The molar concentrations of the peptides were calculated from their proportions in each fraction as determined by gel electrophoresis and staining with Coomassie blue G-250, and from the protein concentrations in the fractions. The absorbance of the gels was recorded and the amounts of the peptides calculated from the areas under the bands. The relative colour indices of the peptides of M_r 79 000, 31 000 and 25 000 were determined to be in the ratio 1 : 0.5 : 0.5 from the contents of [^{3}H]leucine.

of the fractions and the molecular weights. The sulfide eluted in two well-separated bands, the first of which coincided with that of the M_r 79 000 peptide and the second with the M_r 31 000 subunit. The M_r 25 000 peptide contained the cytochrome *b* of which only 20% was recovered, but no sulfide. The sulfide content of the M_r 79 000 peptide was approx. 0.5 mol/mol of peptide and that of the M_r 31 000 peptide approx. 1 mol/mol peptide.

Molecular weight

In equilibrium runs with the analytical ultracentrifuge, the exponential distribution of the cytochrome *b* of fumarate reductase was recorded. The plot of the logarithm of the absorbance against the square of the distance from the center of rotation gave a straight line, confirming the homogeneous nature of the enzyme. From the slope of this line and the partial specific volume, the molecular weight of the enzyme in the complex with Triton and phospholipids was calculated as 326 000 (Table III). On correction for the contents of phospholipids and Triton [27], the molecular weight of the protein of the enzyme containing cytochrome *b* was found to be 200 000. The sedimentation coefficient of this species of the enzyme was 10.5 S as measured with the analytical ultracentrifuge by following the absorbance of cytochrome *b*. A similar value (9.8 S) was obtained by sucrose density gradient centrifugation [20,28] with catalase as the reference protein. In the latter procedure fumarate reductase was detected by its enzymic activity. Because the values of the sedimentation coefficient were consistent, measuring the absorbance of cytochrome *b* reflects the sedimentation behavior of the whole enzyme. The Stokes radius of the enzyme containing cytochrome *b*, as determined by gel filtration, was 6.25 nm. The molecular weights of the complex and of the corresponding protein which were calculated from the sedimentation coefficient and the Stokes radius were 16% greater than those obtained from equilibrium centrifugation.

The sedimentation coefficient of the enzyme lacking cytochrome *b* was obtained from sucrose density gradient centrifugation, and the Stokes radius was determined by gel filtration. The molecular weight of the enzyme complex, as calculated from these two values, was 147 000 and that of the enzyme protein 121 000.

Reactivity of fumarate reductase

Both enzymes catalyzed the reduction of fumarate by viologen radicals or anthrahydroquinonesulfonate as well as the oxidation of succinate by ferricyanide or methylene blue (Table IV). The greatest specific activities were measured with the viologen dyes whilst those of the other reactions were distinctly smaller. Because the specific activities in these reactions were similar in both preparations, these activities are independent of the presence of cytochrome *b* in the enzyme. The enzyme containing cytochrome *b* catalyzed the reduction of oxygen by succinate with H_2O_2 as the product.

The reduction of fumarate by dimethylnaphthohydroquinone or menadiol, as well as the oxidation of succinate by dimethylnaphthoquinone or menadione was catalyzed only by the enzyme containing cytochrome *b*. The activities were independent of the presence of menaquinone in the preparation. The K_m for dimethylnaphthohydroquinone was smaller than that for menadiol. This

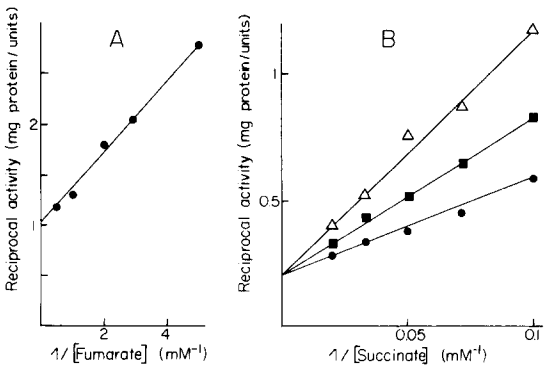


Fig. 6. Activities of fumarate reductase containing cytochrome *b* as functions of the concentrations of fumarate and succinate. (A) Fumarate reduction by menadiol. (B) Succinate oxidation by menadione was measured in the absence of fumarate (●) and with 0.2 mM (■) and 0.5 mM (△) fumarate present.

indicates a certain specificity of the site of naphthohydroquinone reaction for the dimethyl derivative which is structurally more similar to menaquinone. The V with dimethylnaphthohydroquinone is greater than with menadiol. This is probably due to the lower redox potential of dimethylnaphthohydroquinone, since the activity of succinate oxidation is faster with menadione than with dimethylnaphthoquinone as the acceptor.

The activity of fumarate reduction by menadiol and that of succinate oxidation by menadione were related to the concentrations of the substrates according to the Michaelis equation (Fig. 6). The K_m for fumarate was 0.35 mM ($V = 1$ unit/mg protein). A similar value of K_m was measured with benzyl viologen radical as the donor (not shown). The K_m for succinate was 20 mM ($V = 5$ unit/mg protein) with menadione (Fig. 6B), whilst values of approx. 7 mM were measured with ferricyanide or methylene blue as acceptors (not shown). Succinate oxidation by menadione was competitively inhibited by fumarate (Fig. 6B). From the inhibition curves with 0.2 and 0.5 mM fumarate present, the same value of K_i (0.35 mM) was obtained. The identity of this value of K_i with that of K_m for fumarate reduction is consistent with the view that fumarate and succinate interact at the same site of the enzyme.

Discussion

The molecular weights of the two enzyme species as calculated from three independent types of measurement are compared in Table V. The values of the minimal molecular weights which are obtained from the FAD contents and from the peptide composition are nearly the same in both cases. This shows that both enzyme species contain one FAD per set of peptides. From the molecular weights derived from the hydrodynamic properties it follows that the functional enzymes consist of one set of peptides. These results are confirmed by tests on the homogeneity (Fig. 2 and Table III) and on the purity (Fig. 4) of the enzymes. From these and other results presented the following conclusions are drawn.

1. The functional fumarate reductase of *V. succinogenes* is an iron-sulfur

TABLE V

COMPARISON OF THE VALUES OF THE MOLECULAR WEIGHTS OF THE FUMARATE REDUCTASES CALCULATED FROM DIFFERENT EXPERIMENTAL DATA

The FAD values were taken from Table II. The enzyme lacking cytochrome *b* is composed of peptides of M_r 79 000 and 31 000 in the molar ratio of 1:1, and that containing cytochrome *b* of peptides of M_r 79 000, 31 000 and 25 000 in the molar ratio of 1:1:2 (Fig. 4). The values given in the last line were taken from Table III.

M_r calculated from	Cytochrome *b*	
	Absent	Present
FAD content	114 000	167 000
Subunit composition	110 000	160 000
Hydrodynamic properties	121 000	200 000

flavoprotein consisting of an M_r 79 000 and an M_r 31 000 subunit in a molar ratio of 1 : 1.

2. The larger subunit contains the FAD which was previously shown to be linked at position 8α to the N-3 of a histidine of the peptide [15].

3. Both peptides contain iron-sulfur.

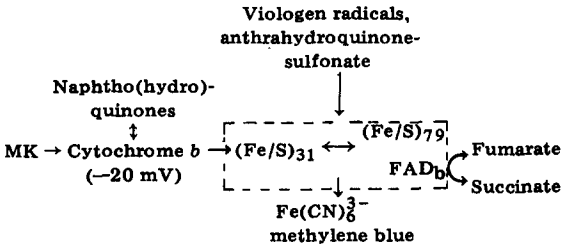
4. The enzyme was derived from a second species which additionally contains 2 mol cytochrome *b* per mol FAD with a molecular weight of 25 000.

From the structural point of view the fumarate reductase of *V. succinogenes* resembles the succinate dehydrogenase of mitochondria [33]. This enzyme also consists of one set of two different peptides, the FAD is covalently linked in the same way [26] to the larger peptide, and both subunits contain iron-sulfur [33]. The succinate dehydrogenase structure of *Rhodospirillum rubrum* [34] and of *Bacillus subtilis* [35] appear to be in accordance with this principle and are also membrane-bound. The catalytic properties of the enzyme of *V. succinogenes* are those of a fumarate reductase and not of a succinate dehydrogenase. The maximum activity of fumarate reduction is two orders of magnitude greater than that of succinate oxidation (Table IV). The K_m for fumarate (0.35 mM) is approximately equal to that of mitochondrial succinate dehydrogenase for succinate, whilst the K_m for succinate is more than 20 times greater.

The cytochrome *b* associated with fumarate reductase consists of two different species. The redox potentials, the spectral properties and the relative proportions (molar ratio 1 : 1) of the cytochromes are the same in fumarate reductase and in the membrane of the bacterium [13]. From the midpoint potential (−200 mV) it is clear that the low-potential cytochrome is not functionally linked to fumarate reductase. As shown earlier, this cytochrome is the primary electron acceptor of formate dehydrogenase [20]. From the inability to separate the two cytochromes by the methods used here and from their identical molecular weights in the presence of sodium dodecyl sulfate, it may be concluded that the molecular properties of the two species are very similar.

Although not required for some of the enzymic activities, cytochrome *b* (−20 mV) is functionally linked to fumarate reductase. This is evident from

its reduction by succinate and from the observation that the activities of fumarate reduction by naphthohydroquinones and those of succinate oxidation by naphthoquinones are dependent on the presence of cytochrome *b*. The activity of fumarate reduction by dimethylnaphthohydroquinone resembles the electron transport from menaquinone to fumarate in the membrane of the bacteria [12,13], because of the similarities of the structures and redox potentials of the two naphthoquinones. Furthermore, the velocity of this reaction is consistent with that of the electron transport catalyzed by the membrane. With saturating amounts of dimethylnaphthohydroquinone present, the turnover number of the FAD of fumarate reductase is approx. $1 \cdot 10^3$ min^{-1} (Tables II and IV). A similar value is obtained for the turnover of the protein-bound FAD of the membrane fraction in the steady state of electron transport from formate to formate [12]. It is concluded that cytochrome *b* (—20 mV) is the primary acceptor of the electrons of menaquinone and the donor to fumarate reductase in the electron transport from menaquinone to fumarate (Scheme I).



Fumarate and succinate interact at the same site of the enzyme. This is indicated by the finding that fumarate is a competitive inhibitor of succinate oxidation and that the K_i is equal to the K_m for fumarate in the fumarate reduction (Fig. 6). Analogous to the situation in the mitochondrial succinate dehydrogenase [36], the substrate site of fumarate reductase is probably localized on the peptide that carries the FAD, and the FAD is probably the redox component which reacts directly with the substrates. The M_r 31 000 peptide is considered to be a functional component of the enzyme and is assumed to mediate the electron transport between cytochrome *b* (—20 mV) and FAD (Scheme I).

Addendum

During the editorial treatment of this paper, two communications on an enzyme from *Escherichia coli* were published by Dickie and Weiner [37,38] which appears to be similar in many respects to the fumarate reductase of *V. succinogenes*. However, as judged from the K_m values the enzyme from *E. coli* is more specific for succinate than for fumarate.

Acknowledgements

The authors thank Dr. H. Aquila and Professor M. Klingenberg for introduction to the procedures of isolation as well as the determination of the molecular weight and the binding of Triton X-100. The technical assistance of

Mrs. M. Heiler and Miss A. König is gratefully acknowledged. M. Vitali kindly corrected the manuscript. The work was supported by a grant of the Deutsche Forschungsgemeinschaft to A.K.

References

1 Kröger, A. (1977) in Microbial Energetics (Haddock, B.A. and Hamilton, W.A., eds.), 27th Symposium of the Society for General Microbiology, pp. 61—93, Cambridge University Press
2 Thauer, R.K., Jungermann, K. and Decker, K. (1977) Bacteriol. Rev. 41, 100—180
3 Kröger, A. (1980) in Diversity of Bacterial Respiratory Systems (C.J. Knowles, ed.), CRC Press Inc., West Palm Beach, U.S.A., in the press
4 Kröger, A. (1978) Biochim. Biophys. Acta 505, 129—145
5 Lara, F.J. (1959) Biochim. Biophys. Acta 33, 565—567
6 Hauber, J. and Singer, T.P. (1967) Eur. J. Biochem. 3, 107—116
7 Warringa, M.P., Smith, O.H., Giuditta, A. and Singer, T.P. (1958) J. Biol. Chem. 230, 97—109
8 Wolin, M.J., Wolin, E.A. and Jacobs, N.J. (1961) J. Bacteriol. 81, 911—917
9 Reddy, C.A. and Peck, H.D. (1978) J. Bacteriol. 137, 982—991
10 Kröger, A., Dorrer, E. and Winkler, E. (1980) Biochim. Biophys. Acta 589, 118—136
11 Kröger, A. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, F., Slater, E.C. and Siliprandi, N., eds.), pp. 265—270, Elsevier/North-Holland
12 Kröger, A. and Innerhofer, A. (1976) Eur. J. Biochem. 69, 487—495
13 Kröger, A. and Innerhofer, A. (1976) Eur. J. Biochem. 69, 497—506
14 Mitchell, P. (1961) Nature 191, 144—148
15 Kenney, W.C. and Kröger, A. (1977) FEBS Lett. 73, 239—243
16 Riccio, P., Aquila, H. and Klingenberg, M. (1975) FEBS Lett. 56, 133—138
17 Klingenberg, M., Riccio, P. and Aquila, H. (1978) Biochim. Biophys. Acta 503, 193—210
18 Von Jagow, G., Schägger, H., Riccio, P., Klingenberg, M. and Kolb, H.J. (1977) Biochim. Biophys. Acta 462, 549—558
19 Von Jagow, G., Schägger, H., Engel, W.D., Machleidt, W. and Machleidt, I. (1978) FEBS Lett. 91, 121—125
20 Kröger, A., Winkler, E., Innerhofer, A., Hackenberg, H. and Schägger, H. (1979) Eur. J. Biochem. 94, 465—475
21 Bernardi, G. (1971) Methods Enzymol. 22, 325—339
22 Bode, C., Goebell, H. and Stähler, E. (1968) Z. Klin. Chem. Klin. Biochem. 6, 419—422
23 Chen, P.S., Toribara, T.Y. and Warnew, H. (1956) Anal. Chem. 28, 1756—1761
24 Brumby, P.E. and Massey, V. (1967) Methods Enzymol. 10, 469—470
25 Neville, D.M. (1971) J. Biol. Chem. 246, 6328—6334
26 Walker, W.H., Singer, T.P., Ghisla, S. and Hemmerich, P. (1972) Eur. J. Biochem. 26, 279—289
27 Tanford, C., Nozaki, Y., Reynolds, J.A. and Makino, S. (1974) Biochemistry 13, 2369—2376
28 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
29 Nozaki, Y., Schlechter, N.M., Reynolds, J.A. and Tanford, C. (1976) Biochemistry 15, 3884—3890
30 Jones, R.W. and Garland, P.B. (1977) Biochem. J. 164, 199—211
31 Clark, W.M. (1960) Oxidation Reduction Potentials of Organic Systems. Williams and Wilkins Co., Baltimore, MD
32 Schnorf, U. (1966) Thesis No. 3871, Eidgenössische Technische Hochschule, Zürich, Switzerland
33 Davis, K.A. and Hatefi, Y. (1971) Biochemistry 10, 2509—2516
34 Davis, K.A., Hatefi, Y., Crawford, J.P. and Baltscheffsky, H. (1977) Arch. Biochem. Biophys. 180, 459—464
35 Hederstedt, L., Holmgren, E. and Rutberg, L. (1979) J. Bacteriol. 138, 370—376
36 Kenney, W.C. and Mowery, P.C. (1976) in Flavins and Flavoproteins (Singer, T.P., ed.), pp. 532—542, Elsevier, Amsterdam
37 Dickie, P. and Weiner, J.H. (1979) Can. J. Biochem. 57, 813—821
38 Weiner, J.H. and Dickie, P. (1979) J. Biol. Chem. 254, 8590—8593